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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/31, C07K 14/35, A61K 39/04, G01N 33/569

A2

(11) International Publication Number: WO 99/45119

(43) International Publication Date: 10 September 1999 (10.09.99)

(21) International Application Number: PCT/DK99/00109

(22) International Filing Date: 5 March 1999 (05.03.99)

(30) Priority Data:

0306/98	6 March 1998 (06.03.98)	DK
60/077,105	6 March 1998 (06.03.98)	US

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(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

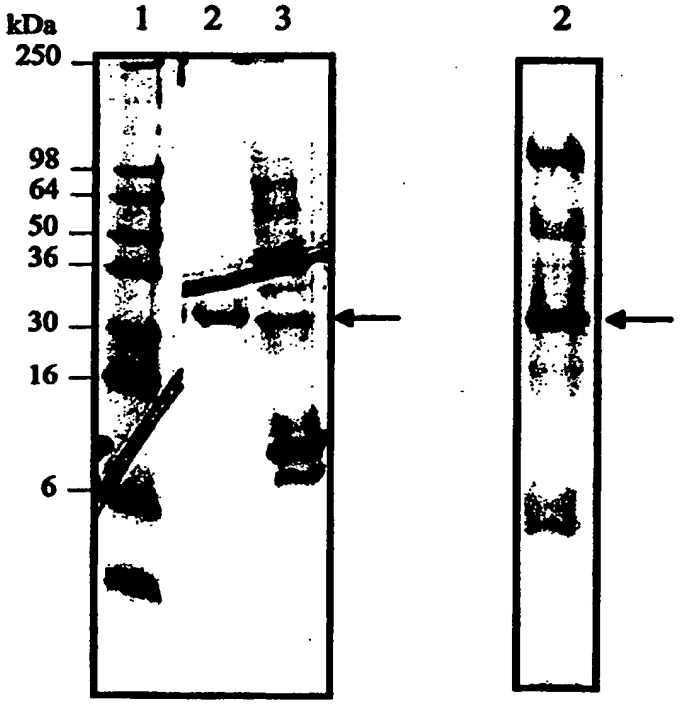
Published

Without international search report and to be republished upon receipt of that report.

(54) Title: PRODUCTION OF MYCOBACTERIAL POLYPEPTIDES BY LACTIC ACID BACTERIA

(57) Abstract

A method of producing in a recombinant lactic acid bacterium such as *Lactococcus lactis* a bioreactive polypeptide derived from mycobacteria belonging to the *M. tuberculosis* complex *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis* or any other mycobacterial species. The lactic acid bacterially produced polypeptide is in the form of monomer or polymers of one or more polypeptide and it is useful as a diagnostic agent in delayed type hypersensitivity (DTH) skin tests in animals and humans and in vaccines. A bioreactive ESAT-6 homodimer polypeptide is described.



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PRODUCTION OF MYCOBACTERIAL POLYPEPTIDES BY LACTIC ACID BACTERIA

FIELD OF INVENTION

The present invention relates to the field of diagnostic tests for tuberculosis and other mycobacterial infections and anti-mycobacterial vaccines, in particular to the provision of polypeptides which are useful as diagnostic reagents in *in vivo* tests and in *in vitro* tests including the delayed type hypersensitivity (DTH) reaction, or as vaccines. In one embodiment of the invention polypeptides are produced in a recombinant lactic acid bacterial host organism and have a high bioreactivity in the DTH reaction and/or the IFN-γ test. In another embodiment of the invention an ESAT-6 homopolymer is produced in any host organism and has a high bioreactivity in the DTH reaction and/or the IFN-γ test.

TECHNICAL BACKGROUND AND PRIOR ART

Tuberculosis remains a major world health problem which is increasing in both developing and industrialised countries. The measures of controlling the disease include early diagnosis and treatment combined with vaccination of the populations at risk. The currently available anti-tuberculosis vaccine was developed in the beginning of this century by Calmette and Guérin and is often referred to as "the Bacille Calmette et Guérin (BCG) vaccine". The vaccine strain was attenuated by serial passages of a virulent isolate of *Mycobacterium bovis* on a bile containing growth medium. The resultant strain appeared to be avirulent for humans. Although the BCG vaccine is widely used, some countries including the USA never introduced it for use in general population vaccination programmes, one reason being that vaccination with BCG interferes with the use of tuberculin skin testing for diagnosing tuberculosis and for use in population surveys.

Infection of susceptible humans or animals with *Mycobacterium tuberculosis* or other mycobacterial species (or vaccination with BCG) will lead to the activation of the cellular immune system. The immunological status of an

individual animal or human being can therefore be monitored by measuring the level of lymphoid cells primed against mycobacterial antigens e.g. by measuring *in vivo* the "delayed type hypersensitivity (DTH) reaction" occurring 24 to 96 hours after intracutaneous injection of mycobacterial antigen.

The product which is currently used in the DTH reaction test is tuberculin, a purified protein derivative (PPD) comprising a crude mixture of *M. tuberculosis* proteins recovered from a synthetic medium where the bacteria has been cultivated for 5 to 6 weeks. However, the structural composition of virulent mycobacteria belonging to the *M. tuberculosis* complex (i.e. *M tuberculosis*, *M. bovis* and *M. africanum*) and the attenuated BCG strain is so closely related that the currently available PPD, due to cross-reactivity, will elicit a positive reaction in a large fraction of vaccinated populations. Thus, in populations that are vaccinated with the BCG vaccine, the currently used tuberculin test cannot distinguish between tuberculosis and immune reactions due to vaccination. Due to the presence of non-pathogenic mycobacteria in the environment in some territories, a false positive reaction with PPD may be observed even though the person is not infected with a pathogenic mycobacterium.

Other antigens have been suggested as possible reagents in a skin test for diagnosing tuberculosis. From WO 92/21697 a diagnostic skin test which comprises a 38 kDa lipoprotein or a 19 kDa protein from *M. tuberculosis* is known. The skin test has specificity for *M. tuberculosis* infections, but also this skin test cannot distinguish between patients immunised with BCG and patients suffering from tuberculosis.

Furthermore, several mycobacterial proteins, e.g. MPT70 and MPT80 from *M. tuberculosis* as well as MPB64 from *M. bovis* have been shown to elicit a DTH reaction in guinea pigs sensitised with mycobacteria belonging to the *M. tuberculosis* complex. The gene encoding MPB64 has been cloned and sequenced (Yamaguchi et al. 1989) from *M. bovis* BCG Tokyo.

In WO 95/01440 is disclosed the *Mycobacterium*-derived polypeptide MPT64 with which lymphoid cells primed with mycobacteria belonging to the *M. tuberculosis* complex are reactive but which polypeptide does not elicit a positive reaction in BCG vaccinated individuals. WO 95/01441 discloses several polypeptides isolated from short-term culture filtrates (ST-CF) of *M. tuberculosis* including the 6-kDa early secretory antigenic target (ESAT-6) antigen which was also shown to be reactive in the DTH test in subjects sensitised with virulent *Mycobacterium* but not in BCG vaccinated individuals.

The coding sequences for both the MPT64 and the ESAT-6 polypeptide have been expressed in *E. coli* (Oettinger et al., 1994; Sørensen et al., 1995). Generally, the group of lactic acid bacteria which for centuries have been used in the manufacturing of food products are considered as safe organisms and more recently, attempts to make use of lactic acid bacterial strains for the production of biologically functional gene products such as pharmaceutically and immunologically active compounds have been reported.

Inducible or regulatable gene expression systems are highly important for expression of genes encoding proteins that are either (i) toxic to the host organism, (ii) needed in large quantities, (iii) used to study the effect of particular gene functions on cellular metabolism or regulation or (iv) produced at a particular point in time or under particular environmental conditions. Whereas inducible expression systems have been developed for use in *E. coli,* only a few inducible expression systems for use in lactic acid bacteria have been described.

One example of an inducible lactic acid bacterial expression system is a system based on the *lac* promoter transcribing the *lac* genes of *Lactococcus lactis*. The *lac* promoter can be repressed by the LacR repressor and a six-fold induction of transcription can be obtained by replacing glucose in the growth medium with lactose (van Rooijen et al., 1992). This naturally occurring expression system has been combined with the T7 RNA polymerase/T7 promoter system from *E. coli* (Wells et al., 1993a,b; Steidler et al., 1995). The

lac promoter controls the expression of T7 RNA polymerase, which recognises the T7 promoter, allowing inducible expression of genes cloned downstream of the T7 promoter. This system has been used to produce tetanus toxin fragment C and murine interleukin-2.

Other examples of lactic acid bacterial inducible expression systems include the use of the *dnaJ* promoter transcribing the *dnaJ* gene of *L. lactis* which has been used to generate inducible expression of a heterologous protein after heat shock induction (van Asseldonk et al., 1993) which results in about four-fold induction of gene transcription and the use of phage specific expression signals from lytic bacteriophages of *L. lactis* which can be applied to express heterologous genes upon phage infection (O'Sullivan et al. 1996)

Further lactic acid bacterial expression systems are systems based on induction of gene expression by supplementing the bacterial growth medium with an inducer substance such as the toxic antitumour antibiotic Mitomycin C (Nauta et al., 1996) or a bacteriocin such as nisin (Ruyter et al., 1996, EP 0712 935 A2).

It has recently been discovered that it is possible to isolate lactic acid bacterial promoters which are inducible or regulatable by the presence/absence or the concentration of one or more environmental factors associated with conventional lactic acid bacterial industrial production methods such as pH, growth temperature, composition of the growth medium including the ionic strength/NaCl content, the presence/absence of purine nucleotide precursors and/or the growth phase/growth rate of the bacterium (WO 94/16086, Israelsen et al., 1995).

It is evident that regulatable expression systems based on such environmental or growth condition factors which are normally present in industrial culture media for lactic acid bacteria either initially or during the cultivation step, represents a highly attractive approach for regulating the production of

heterologous gene products, including mycobacterially derived polypeptides, in lactic acid bacteria.

In order for the application of these regulatable expression systems to be successful, the selected promoter must be effective and lead to the production of a desired protein in sufficiently high amounts under industrial conditions to facilitate an economically viable production or manufacturing process. In this connection it has been found that otherwise useful naturally occurring regulatable lactic acid bacterial promoters may only have a relatively weak promoter activity.

However, it has been discovered by the inventors that the activity of such naturally occurring inducible or regulatable lactic acid bacterial promoters can be increased by modifying the nucleotide region in which the promoter is located and, most importantly, that such an increased promoter activity can be obtained without reducing or eliminating the inducibility by the above mentioned growth condition factors. Additionally, it has also been found that the modification of the promoter region sequences may result in strains having a modulated expression level under induced conditions as compared to the regulation by the corresponding non-modified promoter region.

Thus, there are several gene expression systems available for the production of heterologous polypeptides in lactic acid bacteria. Any of such systems can, in accordance with the present invention, be used for the production of mycobacterial polypeptides, although it is presently preferred to make use of a lactic acid bacterial regulatable gene expression system which does not require the addition of inducing agents or alterations of the growth conditions. In particular, such an expression system which is based on modification of naturally occurring regulatory sequences that are operably linked to the coding sequence for the mycobacterial polypeptide, whereby the expression of the gene can be enhanced significantly, is preferred.

It has surprisingly been found that not only is such a lactic acid bacterial expression system effective in that relatively high yields of the mycobacterial polypeptide is obtained, but the resulting polypeptide has a reactivity in the DTH skin test for tuberculosis which is high and generally higher than the reactivity of the polypeptide when produced in *E. coli* and at substantially the same level as that of the currently used PPD tuberculin.

In an effort to diminish the threat of TB, attenuated bacillus Calmette-Guérin (BCG) has been used as a live attenuated vaccine. BCG is an attenuated derivative of a virulent *Mycobacterium bovis*. The original BCG from the Pasteur Institute in Paris, France, was developed from 1908 to 1921 by 231 passages in liquid culture and has never been shown to revert to virulence in animals, indicating that the attenuating mutation(s) in BCG are stable deletions and/or multiple mutations which do not readily revert. While physiological differences between BCG and *M. tuberculosis* and *M. bovis* have been noted, the attenuating mutations which arose during serial passage of the original BCG strain have been unknown until recently. The first mutations described are the loss of the gene encoding MPB64 in some BCG strains (Li et al., 1993, Oettinger and Andersen, 1994) and the gene encoding ESAT-6 in all BCG strain tested (Harboe et al., 1996), later 3 large deletions in BCG have been identified (Mahairas et al., 1996). The region named RD1 includes the gene encoding ESAT-6 and another region named RD2 includes the gene encoding MPT64. Both antigens have been shown to have diagnostic potential and ESAT-6 has been shown to have properties as a vaccine candidate (cf. WO95/01441 and WO95/01440). The protein ESAT-6 which is present in short-term culture filtrates from mycobacteria as well as the *esat*-6 gene in the mycobacterial genome has been demonstrated to have a very limited distribution in other mycobacterial strains that *M. tuberculosis*, e.g. *esat*-6 is absent in both BCG and the majority of mycobacterial species isolated from the environment, such as *M. avium* and *M. terrae*.

SUMMARY OF THE INVENTION

Accordingly, the invention relates in one aspect to a method of producing a bioreactive polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the tuberculosis complex (*Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*) are capable of reacting, or an analogue that is immunologically equivalent to the polypeptide, the method comprising the steps of (i) providing a DNA sequence coding for said polypeptide, (ii) inserting said coding sequence into a vector capable of replicating in a lactic acid bacterium, (iii) transforming a lactic acid bacterium with said vector, (iv) cultivating the thus transformed bacterium in a culture medium under conditions where the polypeptide is expressed, and (v) harvesting the mycobacterial polypeptide,

the polypeptide thus obtained being capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in at least 10% of guinea pigs previously infected with virulent mycobacteria belonging to the *M. tuberculosis* complex, the DTH reaction essentially being carried out using the following protocol:

(i) an at least partially purified preparation of the polypeptide is diluted with PBS containing 0.005% polysorbate and 0.01% chinisol to obtain a polypeptide concentration of about 20 µg/ml,

(ii) 100 µl of the diluted polypeptide preparation is injected intradermally into a group of Dunkin Hartley guinea pigs infected i.v. 4 weeks prior to the DTH reaction test with the *Mycobacterium tuberculosis* strain H37Rv at a dosage of about 0.5×10^5 CFU, and

(iii) the size of the inflammatory reaction at the sites of injection is measured on day 2 by means of a ruler, a reaction of a size $\geq$ 5 mm being recorded as a positive reaction.

In a further aspect of the invention there is provided a polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the *M.*

tuberculosis complex are capable of reacting, or an analogue that is immunologically equivalent to the polypeptide, the polypeptide is obtainable by the method according to the invention described above and is capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in at least 10% of guinea pigs previously infected with virulent mycobacteria belonging to the *M. tuberculosis* complex, the DTH reaction essentially being carried out using the following protocol:

(i) an at least partially purified preparation of the polypeptide is diluted with PBS containing 0.005% polysorbate and 0.01% chinisol to obtain a polypeptide concentration of about 20 µg/ml,

(ii) 100 µl of the diluted polypeptide preparation is injected intradermally into a group of Dunkin Hartley guinea pigs infected i.v. 4 weeks prior to the DTH reaction test with the *Mycobacterium tuberculosis* strain H37Rv at a dosage of about 0.5×10^5 CFU, and

(iii) the size of the inflammatory reaction at the sites of injection is measured on day 2 by means of a ruler, a reaction of a size $\geq$ 5 mm being recorded as a positive reaction.

In a presently preferred aspect, the present invention relates to a specific polypeptide, the ESAT-6 homopolymer. The ESAT-6 homopolymer can be produced by the method described in the invention, but the ESAT-6 homopolymer product according to the invention can as well be produced by any other method.

In another aspect, there is provided a vector capable of being replicated in a lactic acid bacterial cell, said vector comprising

(i) a promoter region and operably linked hereto

(ii) a DNA sequence coding for a bioreactive polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the *M. tuberculosis* complex are capable of reacting, or an analogue that is immunologically equivalent to the polypeptide, the polypeptide being capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in at least 10% of guinea pigs previously infected with virulent mycobacteria belonging to the *M. tuberculosis* complex, the DTH reaction essentially being carried out using the following protocol:

(i) an at least partially purified preparation of the polypeptide is diluted with PBS containing 0.005% polysorbate and 0.01% chinisol to obtain a polypeptide concentration of about 20 µg/ml,

(ii) 100 µl of the diluted polypeptide preparation is injected intradermally into a group of Dunkin Hartley guinea pigs infected i.v. 4 weeks prior to the DTH reaction test with the *Mycobacterium tuberculosis* strain H37Rv at a dosage of about 0.5×10^5 CFU, and

(iii) the size of the inflammatory reaction at the sites of injection is measured on day 2 by means of a ruler, a reaction of a size $\geq$ 5 mm being recorded as a positive reaction.

In a still further aspect, the invention pertains to a lactic acid bacterium which is transformed with the vector according to the invention.

The invention provides in further aspects a method of producing a bioreactive polypeptide which is derived from a *Mycobacterium* not belonging to the *M. tuberculosis* complex, the method comprising the steps of (i) providing a DNA sequence coding for said polypeptide, (ii) inserting said coding sequence into a vector capable of replicating in a lactic acid bacterium, (iii) transforming a lactic acid bacterium with said vector, (iv) cultivating the thus transformed bacterium in a culture medium under conditions where the polypeptide is expressed, and (v) harvesting the mycobacterial polypeptide, said polypeptide

being selected from the group consisting of a polypeptide with which lymphoid cells previously primed with mycobacteria are capable of reacting and an analogue of said polypeptide that is immunologically equivalent to the polypeptide, a polypeptide that is obtainable by said method, a diagnostic composition and a vaccine comprising a polypeptide according to invention.

DETAILED DISCLOSURE OF THE INVENTION

A major objective of the present invention is to provide a bioreactive polypeptide or an immunologically equivalent analogue hereof which in the delayed type hypersensitivity skin test for tuberculosis has a reactivity which is of the same order as the reactivity of the currently used PPD tuberculin reagent, gives rise to a higher number of true positive reactions than the PPD, and is produced in a GRAS recombinant host organism in high yields and with a structural configuration that does not impair the bioreactivity of the polypeptide.

In the present context, the term "bioreactivity" refers i.a. to the capability of the polypeptide to elicit a DTH skin reaction in a human being or an animal that has previously been exposed to virulent mycobacteria of the *M. tuberculosis* complex or to other mycobacterial species. In the standard DTH skin test an effective amount of the test reagent is administered into a site of the human being or animal and the DTH reaction typically measured 24 to 96 hours after exposure to the test reagent. The mode of administration can vary and includes intracutaneous (intradermal) injection, subcutaneous injection, introduction by means of high pressure air and administration by means of a sharp-pointed instrument or set of such instrument (multi-puncture). A positive reaction is defined as the diameter of the erythematous skin reaction at the site of administration. A positive reaction may e.g. be defined as an erythema diameter of $\geq$ 5 mm.

Preferably, the bioreactivity of the polypeptide is specific for tuberculosis in the sense that it is a polypeptide with which lymphoid cells previously primed with mycobacteria of the *M. tuberculosis* complex or other mycobacteria are capable of reacting and with which lymphoid cells previously primed with a BCG vaccine are not capable of reacting. Examples of currently used BCG vaccines include *Mycobacterium bovis* BCG Copenhagen, also designated "BCG strain: Danish 1331", *M. bovis* BCG Glaxo, *M. bovis* BCG Pasteur, *M. bovis* BCG Canadian and *M. bovis* BCG.

In accordance with the invention it may also be advantageous that the polypeptide in addition to being specific for tuberculosis as defined above is specific in the sense that it very rarely reacts with lymphoid cells which are primed by other organisms including mycobacteria which do not belong to the *M. tuberculosis* complex. Such mycobacteria not belonging to the *M. tuberculosis* complex include the group consisting of *M. avium, M. intracellulare, M. kansasii, M. scrufolaceum, M. marinum, M. ulcerans, M. fortuitum, M. chelonei, M. phlei, M. gordanae, M. smegmatis, M. paratuberculosis* and *M. leprae*.

The bioreactivity of a polypeptide according to the invention may also be determined by *in vitro* tests wherein the polypeptide is reacted with lymphoid cells or a body fluid such as a blood sample, or another type of sample containing such cells. Thus, the bioreactivity can be expressed as the capability of the polypeptide to stimulate lymphoid cells to release IFN-γ. In other words, the bioreactive polypeptide induces a release of IFN-γ from primed memory T-lymphocytes withdrawn from a mouse within 2 weeks of primary infection or within 4 days after the mouse has been re-challenge infected with mycobacteria belonging to the tuberculosis complex, the induction being performed by the addition of the polypeptide to a suspension comprising about 200,000 spleen cells per ml, the addition of the polypeptide resulting in a concentration of 1-4 μg polypeptide per ml suspension, the release of IFN-γ being assessable by determination of IFN-γ in supernatant harvested 2 days after the addition of the polypeptide to the suspension.

In a preferred embodiment the bioreactive polypeptide is specific. One way of defining a specific bioreactive polypeptide is a polypeptide that induces a release of IFN-γ of at least 1,500 pg/ml above background level from about 1,000,000 human PBMC (peripheral blood mononuclear cells) per ml isolated from TB patients in the first phase of infection, or from healthy BCG vaccinated donors, or from healthy contacts to TB patients, the induction being performed by the addition of the polypeptide to a suspension comprising

the about 1,000,000 PBMC per ml, the addition of the polypeptide resulting in a concentration of 1-4 μg polypeptide per ml suspension, the release of IFN-γ being assessable by determination of IFN-γ in supernatant harvested 2 days after the addition of the polypeptide to the suspension.

Preferably, in the 2 IFN-γ assays the release effected by the bioreactive polypeptide gives rise to at least 1,500 pg/ml IFN-γ in the supernatant but higher concentrations are preferred, e.g. at least 2,000 pg/ml and even at least 3,000 pg/ml IFN-γ in the supernatant. The IFN-γ release from bovine PBMC can e.g. be measured as the optical density (OD) index over background in a standard cytokine ELISA and should thus be at least two, but higher numbers such as at least 3, 5, 8, and 10 are preferred.

It is presently contemplated that when this application refers to IFN-γ release as a measure of bioractivity, other cytokines could be relevant, such as IL-12, TNF-α, IL-4, IL-5, IL-10, IL-6, TGF-β. Usually one or more cytokines will be measured utilising for example the PCR technique or ELISA. It will be appreciated by the person skilled in the art that a significant increase or decrease in any of these cytokines will be indicative of an bioractive polypeptide or polypeptide fragment.

Additionally, the bioreactivity can be associated with the ability of the polypeptide to elicit the formation of antibodies in an organism into which the polypeptide is introduced. Such an ability can be determined by testing a sample from that organism for the presence of antibodies reactive with the particular polypeptide using any conventional method for detecting antibodies.

As used herein, the term "polypeptide" is used to indicate both short peptides with a length of at least two amino acid residues and at most 10 amino acid residues, oligopeptides (11-100 amino acid residues, such as at most 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 or at most 95 amino acid residues), and longer peptides (the usual interpretation of "polypeptide", i.e. more than 100 amino acid residues in length, such as

more than 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625 or more than 650 amino acid residues) as well as proteins (the functional entity comprising at least one peptide, oligopeptide, or polypeptide which may be chemically modified by being glycosylated, by being acylated or by comprising prosthetic groups). The definition of polypeptides also encompasses native forms of peptides/proteins occurring naturally in mycobacteria as well as recombinant proteins or peptides in any type of expression vectors transforming any kind of lactic acid bacterial host cells and also chemically synthesised peptides.

In one embodiment of the invention antigens such as CFP7, CFP7A, CFP7B, CFP8A, CFP8B, CFP9, CFP10A, CFP11, CFP17, CFP19, CFP19A, CFP19B, CFP20, CFP21, CFP22, CFP23, CFP23A, CFP23B, CFP25, CFP25A, CFP27, CFP28, CFP29, CFP30A, CFP50, MPT51, CWP32, RD1-ORF8, RD1-ORF2, RD1-ORF9B, RD1-ORF3, RD1-ORF9A, RD1-ORF4, RD1-ORF5, MPT59-ESAT6, and/or ESAT6-MPT59 are produced. The antigens are described in WO98/44119, hereby incorporated by reference.

The term "immunologically equivalent analogue" is used herein to indicate a variant, analogue or subsequence of a polypeptide of the invention that is functionally equivalent to a naturally occurring mycobacterial polypeptide with respect to its ability to elicit a DTH reaction or its reactivity in the IFN-γ assay, provided the DTH reaction is of the same order or to an extent of at least 10% of the DTH reaction elicited by the polypeptide under the same conditions, such as at least 20%, e.g. at least 30% including at least 50%, measured as the diameter of the DTH skin reaction. Even higher DTH eliciting ability is contemplated such as at least 60%, including at least 75%, e.g. at least 90%, measured as the diameter of the DTH skin reaction. Likewise, the reactivity of the immunologically equivalent analogue in the IFN-γ assay is of the same order or to an extent of at least 10% of the reactivity elicited by the polypeptide under the same conditions, such as at least 20%, e.g. at least 30% including at least 50%.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations thereof such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The method according to the invention of producing a bioreactive polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the tuberculosis complex (*Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*) are capable of reacting, comprises as a first step the provision of a DNA sequence coding for the polypeptide or an immunologically equivalent analogue, variant or subsequence hereof. The DNA sequence can be derived from any mycobacterial species that produces a polypeptide as defined above using methods which are generally known in the art and examples of which are described in the following examples. Thus, the DNA sequence can be isolated from a species of the *M. tuberculosis* complex or from any other mycobacterial species including those mentioned above.

The DNA sequence may also be a DNA fragment comprising a subsequence, analogue or a variant of a naturally occurring DNA sequence, the subsequence, analogue or variant encoding a polypeptide which is immunologically equivalent to a naturally occurring polypeptide of mycobacterial origin.

By the terms "analogue" or "variant" with regard to the DNA sequences of the invention is understood a nucleotide sequence that encodes a polypeptide exhibiting identical or substantially identical immunological properties to a naturally occurring mycobacterial polypeptide.

It is well known that the same amino acid may be encoded by different codons, the codon usage i.a. being related to the preference of the organisms in question expressing the nucleotide sequence. Thus, one or more nucleotides

or codons of a DNA sequence according to the invention may be exchanged by others which, when expressed, result in a polypeptide identical or substantially identical to the polypeptide encoded by the DNA fragment in question.

Therefore, the terms "analogue" and "variant" are used in the present context to indicate a DNA sequence of a similar nucleotide composition or sequence as the DNA sequence encoding a naturally occurring polypeptide amino acid sequence, allowing for minor variations which do not have an adverse effect on the ligand binding properties and/or biological function and/or immunogenicity as compared to the naturally occurring polypeptide or which give interesting and useful novel binding properties or biological functions and immunogenicities relative to the naturally occurring polypeptide. The analogous DNA fragment or DNA sequence may be derived from an animal or a human or may be partially or completely of synthetic origin as described above. The analogue may also be derived through the use of recombinant DNA techniques.

Furthermore, the terms "analogue" and "subsequence" are intended to allow for variations in the sequence such as substitutions, insertions (including introns), additions, deletions and rearrangements of one or more nucleotides, which variations do not have any substantial effect on the polypeptide encoded by a DNA sequence or a subsequence thereof. The term "substitution" is intended to mean the replacement of one or more nucleotides in the full nucleotide sequence with one or more different nucleotides, "addition" is understood to mean the addition of one or more nucleotides at either end of the full nucleotide sequence, "insertion" is intended to mean the introduction of one or more nucleotides within the full nucleotide sequence, "deletion" is intended to indicate that one or more nucleotides have been deleted from the full nucleotide sequence whether at either end of the sequence or at any suitable point within it, and "rearrangement" is intended to mean that two or more nucleotide residues have been exchanged with each other.

In the present context, a variant or analogue of a DNA sequence is preferably one that hybridises under stringent hybridisation conditions which are to be understood in their conventional meaning i.e. the hybridisation is carried out at 65°C in 2xSSC and final washing at 65°C in 1xSSC.

In accordance with the invention, the DNA sequence may be of cDNA or genomic origin as discussed above, but it can also be of synthetic origin. Furthermore, the DNA sequence may be of mixed cDNA and genomic, mixed cDNA and synthetic or genomic and synthetic origin as discussed above. The DNA sequence may have been modified, e.g. by site-directed mutagenesis, to result in the desired DNA fragment encoding the desired polypeptide. The following discussion focusing on modifications of DNA encoding the polypeptide should be understood to encompass also such possibilities, as well as the possibility of constructing the DNA sequence by ligation of two or more DNA fragments to obtain the desired DNA fragment, and combinations of the above-mentioned principles.

The DNA sequence may be modified using any suitable technique which results in the production of a DNA fragment encoding a polypeptide of the invention. However, any modification of the DNA sequence encoding the amino acid sequence of the polypeptide of the invention should be one which does not impair the immunological functionality of the resulting polypeptide.

In accordance with on of the methods of the invention, the coding DNA sequence as defined above is inserted into a vector capable of replicating in a lactic acid bacterium and a lactic acid bacterium is transformed with the expression vector. The vector may be any vector which can be subjected to recombinant DNA procedures and the choice of vector may depend on the particular lactic acid bacterial host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector i.e. a vector that exists as an extrachromosomal entity, the replication of which is independent of replication of the host cell chromosome. Examples of such a vector are a

plasmid, phage, cosmid, mini-chromosome or virus. Alternatively, the vector may be one which, when introduced in a host cell, is integrated in the host cell genome and replicated together with the chromosome into which it has been integrated. The methods used to construct the vector and to effect its introduction into the host cells can be any methods known in the field of recombinant DNA technology for these purposes.

The polypeptides of the invention are excellent candidates for vaccine constituents or as constituents in an immune diagnostic agent. Thus, another part of the invention pertains to an immunologic composition comprising a polypeptide or a homopolymer of a polypeptide according to the invention. In order to ensure optimum performance of such an immunologic composition it is preferred that it comprises an immunologically and pharmaceutically acceptable carrier, vehicle or adjuvant. Such an immunologic composition may preferably be in the form of a vaccine or in the form of a diagnostic agent e.g. a skin test reagent or an *in vitro* diagnostic reagent.

Suitable carriers are selected from the group consisting of a polymer to which the polypeptide(s) is/are bound by hydrophobic non-covalent interaction, such as a plastic, e.g. polystyrene, or a polymer to which the polypeptide(s) is/are covalently bound, such as a polysaccharide, or a polypeptide, e.g. bovine serum albumin, ovalbumin or keyhole limpet haemocyanin. Suitable vehicles are selected from the group consisting of a diluent and a suspending agent. The adjuvant is preferably selected from the group consisting of dimethyldi-octadecylammonium bromide (DDA), Quil A, poly I:C, Freund's incomplete adjuvant, IFN-γ, IL-2, IL-12, monophosphoryl lipid A (MPL), and muramyl dipeptide (MDP).

In line with the above, the invention also pertains to a method for producing an immunologic composition according to the invention, the method comprising preparing, synthesising or isolating a polypeptide according to the invention, and solubilising or dispersing the polypeptide in a medium for a

vaccine, and optionally adding other *M. tuberculosis* antigens and/or a carrier, vehicle and/or adjuvant substance.

Preparation of immunological compositions, such as vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such immunological compositions, such as vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the immunological compositions, such as vaccines .

The immunological compositions, such as vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly or may even be applied directly on the skin. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral or nasal formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The polypeptide may be formulated into the immunological compositions, such as vaccines as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The immunological compositions, such as vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically and/or diagnostically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 µg to 1000 µg, such as in the range from about 1 µg to 300 µg, and especially in the range from about 10 µg to 50 µg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral or nasal application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccines will depend on the route of administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated and/or tested.

Various methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. According to the invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant, but also Freund's complete and incomplete adjuvants as well as QuilA and RIBI are interesting possibilities. Further possibilities are monophosphoryl lipid A (MPL), and muramyl dipeptide (MDP). Other possibilities involve the use of immune modulating substances such as lymphokines (e.g. IFN-γ, IL-2 and IL-12) or synthetic IFN-γ inducers such as poly I:C in combination with the above-mentioned adjuvants.

In many instances, it will be necessary to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain the desired levels of protective immunity. The course of the immunization may be followed by *in vitro* proliferation assays of PBL (peripheral blood lymphocytes) co-cultured with ESAT-6 or ST-CF, and especially by measuring the levels of IFN-γ released form the primed lymphocytes. The assays may be performed using conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These techniques are well known and may be found in a wide variety of

patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise several different polypeptides in order to increase the immune response. The vaccine may comprise two or more polypeptides, where all of the polypeptides are as defined above, or some but not all of the peptides may be derived from a bacterium belonging to the *M. tuberculosis* complex. In the latter example the polypeptides not necessarily fulfilling the criteria set forth above for polypeptides may either act due to their own immunogenicity or merely act as adjuvants. Examples of such interesting polypeptides are MPB64, MPT64, and MPB59, but any other substance which can be isolated from mycobacteria are possible candidates.

One reason for admixing the polypeptide(s) of the invention with an adjuvant is to effectively activate a cellular immune response. However, this effect can also be achieved in other ways, for instance by expressing the effective antigen in a vaccine in a non-pathogenic microorganism. A well-known example of such a microorganism is *Mycobacterium bovis* BCG.

Therefore, another aspect of the present invention is an improvement of the living BCG vaccine presently available, which is a vaccine for immunising an animal, including a human being, against TB caused by mycobacteria belonging to the tuberculosis-complex, comprising as the effective component a microorganism, wherein one or more copies of a DNA sequence encoding a polypeptide as defined above has been incorporated into the genome of the microorganism in a manner allowing the microorganism to express and secrete the polypeptide.

Yet another aspect of the present invention is a vaccine for immunising an animal, including a human being, against tuberculosis caused by mycobacteria belonging to the tuberculosis complex, comprising as the effective component

one or more copies of a nucleotide sequence, such as a DNA sequence, encoding a polypeptide as defined above, such as an ESAT-6 homopolymer. The vaccine comprising one or more copies of a nucleotide sequence may be introduced into the mammalian sell by e.g. intramuscular injection or percutaneous administration (the so-called "gene gun" approach). The nucleotide sequence is taken up by e.g. muscle cells and the polypeptide of interest is expressed by a promoter which is functioning in mammalian cells, e.g. a viral promoter. Thereafter the polypeptide product stimulates the immune system and gives rise to an immune response against the polypeptide.

The use of the gram-negative bacterium *E. coli* for production of bioreactive mycobacterial polypeptides including tuberculosis skin test reagents may be objectionable for safety reasons. There is therefore a need for alternative production organisms which have GRAS (generally recognised as safe) status. Additionally, in the *E. coli* expression system the mycobacterial polypeptides are located intracellularly and the process of isolating them from the host cells may result in conformational or structural changes that result in a reduced bioreactivity. However GRAS organisms within the *E. coli* family such as the K12 strain and GRAS host organisms such as yeast, e.g. *Pischia pastoris* and *Saccharomyces cerevisiae* are anticipated to express the ESAT-6 homopolymer in way suitable for purification.

As used herein, the term "lactic acid bacterium" designates a gram-positive, microaerophilic or anaerobic bacterium which ferments sugars with the production of acids including lactic acid as the predominantly produced acid, acetic acid and propionic acid. The industrially most useful lactic acid bacteria are found among *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp. and *Propionibacterium* spp. Additionally, lactic acid producing bacteria belonging to the group of the strict anaerobic bacteria, bifidobacteria, i.e. *Bifidobacterium* spp., which are frequently used as food starter cultures alone or in combination with lactic acid bacteria, are generally included in the group of lactic acid bacteria. A presently preferred host cell species is *Lactococcus lactis*.

Following the transformation of the selected lactic acid bacterial host species, the transformed bacterium is cultivated in a culture medium under conditions where the polypeptide is expressed. The culture medium used to cultivate the recombinant lactic acid bacterial host cells can be any conventional medium which is suitable for the purpose e.g with respect to its nutrient composition and pH. In useful embodiments, the host cells are cultivated under anaerobic conditions in an industrial production scale. In the present context, "industrial production scale" indicates that the volume of culture medium in the fermentation vessel is at least 1 L, such as at least 5 L e.g. at least 10 L. It is also envisaged that the volume can be larger such as at least 100 L including at least 250 L.

The choice of specific fermentation conditions such as the fermentation time and temperature depends on the requirements of the selected lactic acid bacterial host cell. Generally, the fermentation time is in the range of 10 to 30 hours such as in the range of 20 to 30 hours.

Preferably, the amount of polypeptide that is secreted into the culture medium after completion of the lactic acid bacterial fermentation process is at least 20 mg/L, such as at least 50 mg/L, preferably at least 100 mg/L e.g at least 250 mg/L including at least 500 mg/L.

In a final step of the method according to the invention, the polypeptide is harvested. Depending on whether or not the coding sequence is associated with a signal sequence which effects the secretion of the polypeptide across the cell membrane and into the culture medium, the step of harvesting includes either the isolation of the polypeptide from the host cells (no signal sequence) or that it is isolated directly from the culture medium. These steps can be carried out using any conventional method for isolating polypeptides. Thus, when the polypeptide is secreted into the culture medium, the first step of harvesting is a separation of the host cells e.g. by centrifugation or filtration followed by isolating the polypeptide from the supernatant or the filtrate. It is

preferred that the polypeptide amounts to at least 25% of the total protein content of the supernatant or the filtrate such as at least 30%, including at least 40%, e.g. at least 50%.

Generally, the supernatant or the filtrate is subjected to a step of concentration and/or at least partial purification using any conventional method for such purposes such as e.g. cross-flow filtration, salting out, immunoaffinity chromatography, hydrophobic interaction chromatography and/or ion exchange chromatography. In preferred embodiments, the concentrated and at least partially purified preparation of the polypeptide contains at least 0.5 mg/ml of polypeptide, such as at least 1.0 mg/ml including at least 1.5 mg/ml e.g. at least 2.0 mg/ml.

Generally, it is preferred that the polypeptide is secreted into the culture medium rather than being accumulated intracellularly, as it appears that a polypeptide that is not subjected to extraction from the host cells may have a higher bioreactivity than a cell-extract derived polypeptide.

The crude or optionally partially purified polypeptide preparation obtained by the harvesting step as defined above may be used as such or it may be formulated to provide a storage stable and convenient skin test diagnostic composition. Thus, such a ready-to-use composition may e.g. include preserving agents, polypeptide stabilising agents or substances which enhance the bioreactivity of the polypeptide. Additionally, a crude polypeptide preparation may be subjected to further concentration or dilution in order to obtain a pre-determined amount or activity of the ready-to-use diagnostic composition.

It is also possible to modify the polypeptide produced by the method of the invention to achieve a diagnostic reagent or vaccine component having, relative to the polypeptide produced, an enhanced bioreactivity in a DTH skin tests, the IFN-γ tests or an enhanced immunogenic potency. A positive skin test response is only obtained if a sufficient amount of the polypeptide

remains at the location of injection. However, the size of some polypeptides may be so small that the polypeptide diffuses rapidly in the extracellular compartment at the site of injection resulting in a less effective skin response. Consequently, the polypeptide may be used as the basis for constructing a homopolymer or a heteropolymer of the polypeptide, whereby the polypeptide does not diffuse freely in the extracellular compartment and is effectively taken up by antigen-presenting cells at the site of injection.

A "homopolymer" of the polypeptide is to be understood in its usual meaning i.e. a polymer formed by two or more identical polypeptides, whereas a "heteropolymer" may be formed from at least two different polypeptides or formed from a polypeptide and a heterologous carrier molecule. A homopolymer may e.g. comprise 2 or more copies of the polypeptide such as 2-20 copies or 2-10 copies including 2-6 copies such as 3, 4, or 5 copies.

An example of the synthesis of a homopolymer may be the introduction of one or more N-terminal cysteine residues in the polypeptide, thereby allowing the homopolymer to be formed as a result of intermolecular disulphide bridges.

The synthesis of a heteropolymer may be carried out by coupling the polypeptide to a different mycobacterial polypeptide such as the mycobacterial protein MPT59 (Nagai et al., 1991) or a part thereof.

By the synthesis of a polymer of the polypeptide the specific activity or potency will increase because the polypeptide will not diffuse freely in the extracellular compartment, whereby a smaller dose of the polypeptide is necessary to elicit an observable DTH reaction or IFN-γ release.

A specific example of a homopolymer is illustrated in the examples with an ESAT-6 homodimer (SEQ ID NO: 2). In one embodiment of the invention the dimer comprises, apart from the two copies of ESAT-6, two features: a linker sequence and an N-terminal leader sequence.

The linker sequence, that is the chemical entity bridging the copies of ESAT-6, is any substance capable of keeping at least two copies of a polypeptide together. In one embodiment the linker sequence consists of amino acids such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 amino acids. In a preferred embodiment the linker sequence consists of 2 amino acids such as Arg-Ser (SEQ ID NO: 3). In another embodiment the ESAT-6 proteins are positioned directly next to each other. In yet another embodiment the ESAT-6 proteins are chemically assembled e.g. on a lysine tree or other chemical backbones, the ESAT-6 proteins are kept together as a result of intermolecular disulphide bridges by adding cysteine residues in the polypeptide, or the ESAT-6 proteins are kept together as a result of intermolecular binding between lysine residues in the ESAT-6 protein and biotin.

Aiming at authorisation of the medical product comprising the ESAT-6 homodimer, the number of potential artificial epitopes in the product should be limited. It is generally accepted that a T-cell epitope is more than 6 amino acids. Thus, in order to avoid the linker sequence to be immunologically relevant, it is preferred that the linker sequence is less than 6 amino acids.

The content of the N-terminal leader sequence preceding ESAT-6 should be chosen such that it does not interfere with the homopolymer in the final product. That is, it should not physically cover epitopes or interfere with the conformation of the product. Furthermore, the N-terminal leader sequence inhibits secretion if it is substantially polar. Thus, in one embodiment of the invention, the N-terminal leader sequence is omitted, or limited to a few amino acids, such as 6, 5, 4, 3, 2, 1 amino acids. In a preferred embodiment, the N-terminal leader sequence is a sequence such as the Asp-Thr-Arg-Ser sequence (SEQ ID NO: 4). It is anticipated that the purification of the product could be eased if the N-terminal leader sequence constitutes a handle e.g. an amino acid sequence capable of reacting with an antibody.

The bioreactivity of the ESAT-6 homodimer is characterised by a high sensitivity. That is the true positive responders. It is calculated from a sample

of animals positively infected with tuberculosis. The number of animals from that sample that reacts with the polypeptide of the invention determines the sensitivity of the polypeptide. For example, if 100 animals have a positive diagnosis of tuberculosis, and 89 of the animals reacts with the polypeptide, the sensitivity of the polypeptide is 89%.

The bioreactivity of the ESAT-6 homodimer is also characterised by a high specificity. The specificity is related to the number of true negative responders. It is calculated from a sample of animals vaccinated with a non-virulent strain of mycobacterium e.g. BCG Danish. The number of animals from that sample that does not react with the polypeptide of the invention determines the specificity of the polypeptide. For example, if 100 animals have been vaccinated against tuberculosis, and 8 of the animals reacts with the polypeptide, the specificity of the polypeptide is 92%.

In the present context the term "cut-off value" refers to the minimal signal from the test which is regarded as a positive signal. Therefore, apart from the immunological nature of the polypeptide used in the test, also the cut-off value used in the test has an impact on the sensitivity and specificity of an assay. If e.g. the cut-off value is set to a very low value of the measured parameter (e.g. the diameter of the skin test reaction), the sensitivity of the assay will approximate 100 but on the expense of specificity which will decrease, as the true negatives will be deemed positive in the test if just a slight reaction is induced.

It will therefore be understood that the efficacy of a given test is highly dependent on the cut-off value and that the determination of the cut-off value further is dependent on the intended use of the test. Of course, the normal situation is that the test should be both sensitive and specific, but under some circumstances this need not be imperative. This can e.g. be the case in situations where a sensitive screening test is used to narrow the "field of search" and one or more specific verification test(s) is/are used to verify the result of the screening test. In this situation, the first screening test need not

be very specific, and accordingly the verification test need not be very sensitive if the verification step, taken as a whole, has the same sensitivity as the screening test.

In this application the term ESAT-6 is characterised by the specific amino acid (SEQ ID NO: 6) and nucleic acid sequence (SEQ ID NO: 5). It will be understood that the invention includes analogues and variants of these sequences produced by recombinant methods wherein the ESAT-6 nucleic acid sequence have been modified by substitution, insertion, addition and/or deletion of one or more nucleotides in said nucleic acid sequence to cause the substitution, insertion, addition or deletion of one or more amino acid residues in the ESAT-6 recombinant polypeptide. In both immunodiagnostics and vaccine preparation, it is often possible and practical to prepare antigens from segments of a known immunogenic protein or polypeptide. Certain epitopic regions may be used to produce responses similar to those produced by the entire antigenic polypeptide. Potential antigenic or immunogenic regions may be identified by any of a number of approaches, e.g., Jameson-Wolf or Kyte-Doolittle antigenicity analyses or Hopp and Woods (1981) hydrophobicity analysis (see, e.g., Jameson and Wolf, 1988; Kyte and Doolittle, 1982; or U.S. Patent No. 4,554,101). Further, in order to identify relevant T-cell epitopes which are recognised during an immune response, it is also possible to use a "brute force" method: Since T-cell epitopes are linear, deletion mutants of ESAT-6 reveal which regions of the polypeptides are essential in immune recognition, e.g. by subjecting these deletion mutants to the IFN-γ assay described herein. Another method utilises overlapping oligomers (preferably synthetic oligomers having a length of e.g. 20 amino acid residues) derived from ESAT-6. At least some of these will give a positive response in the IFN-γ assay whereas others will probably not.

Other kinds of modifications of the polypeptide may be relevant in order to increase its activity. Such modifications may be post-translational modifications such as acylation i.e. addition of a lipid moiety and/or glycosylation.

The relevant functional parts of the polypeptide with respect to the ability of the polypeptide to elicit a DTH reaction are the lymphoid cell epitopes, i.e. the parts of the amino acid sequence that are recognised by lymphoid cells. These epitopes may either be linear or structural.

The injection of the polypeptide as described herein may lead to an undesired sensitisation of individuals diagnosed for tuberculosis if the same individual is subjected to the skin test more than once, or in extreme situations more than twice.

Accordingly, it may desirable that the polypeptide has been modified in order to abolish or delete sensitising epitopes, without abolishing the epitopes that are relevant with respect to the DTH reactions or the IFN-γ test. This can be carried out by several methods well-known to the person skilled in the art. One method may be to modify the polypeptide by denaturing procedures, such as a heat treatment or a formaldehyde treatment.

Alternatively, the nucleotide sequence encoding the polypeptide can be modified in such a way that the translated amino acid sequence lacks all or some of the sensitising epitopes. In the present context the expression "sensitising epitopes" means epitopes that cause sensitisation of a person when the skin test has been used for diagnostic purposes. These epitopes may be either B-cell epitopes or T-cell epitopes.

A polypeptide as defined above is capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in a human being or an animal to the extent as it is defined above or is capable of eliciting an IFN-γ release as defined above. In general, the skin DTH reaction is determined by visual inspection of the skin into which the polypeptide has been injected and by the use of a ruler. A positive skin test is generally between 5 and 40 mm, most often between 10 and 30 mm in diameter such as 5, 6, 7, 8, 9, 10, 11, 12, 15, 20 or 25 mm. Specifically, the polypeptide provided herein is capable of eliciting such a

reaction in at least 10% of guinea pigs previously infected with virulent mycobacteria belonging to the *M. tuberculosis* complex when the DTH reaction being is carried out using the protocol defined herein and as described in details in the following examples.

In a useful embodiment of the invention, the polypeptide is one derived from *Mycobacterium tuberculosis*. Examples of naturally occurring *M. tuberculosis* polypeptides are described e.g. in WO 95/01440 and WO 95/01441 herein incorporated by reference. For the reasons stated above, it is preferred that the coding sequence for such a polypeptide is operably linked to a signal sequence such that the polypeptide is secreted across the cell membrane.

In another useful embodiment, the method provides a *M. tuberculosis*-derived polypeptide which is an immunologically active polypeptide carrying an epitope or an antigenic determinant, said polypeptide being capable of conferring immunity to infections with a bacterium of the *M. tuberculosis* complex when administered in an immunologically effective amount to an animal including a human being. Thus, the polypeptides provided by the invention are also highly useful as vaccines.

It will be appreciated that the term "immunity" does not imply that it is a requirement that a 100% immunity to infection is obtained in the sense that an individual is completely protected against the infection or that 100% of a vaccinated population acquires immunity. It is well-known in the art that vaccines are generally not capable of conferring a 100% immunity.

As it has been mentioned, it is considered advantageous to provide the polypeptide in the form of a homopolymer or a heteropolymer. Accordingly, in a useful embodiment, the DNA sequence coding for the polypeptide is part of a hybrid coding sequence whereby the polypeptide is expressed as part of a fusion protein, including a sequence that codes for a fusion protein, comprising at least two polypeptides of mycobacterial origin which e.g. may have essentially the same amino acid sequence. As one example, such a

fusion protein comprises at least two polypeptides of *Mycobacterium tuberculosis* origin, e.g. at least two polypeptides of *Mycobacterium tuberculosis* origin having essentially the same amino acid sequence. The coding sequence for such a fusion protein is preferably operably linked to a sequence coding for a signal peptide to provide secretion of the fusion protein across the cell membrane.

It is contemplated that the efficacy of the polypeptide as provided herein is reduced if it is in a form where it contains a significant proportion of "irrelevant" amino acids which are not naturally associated with the polypeptide. Thus it is considered advantageous that the polypeptide molecule as harvested is in a form where it contains less than 10% amino acids which are not naturally associated with the polypeptide, such as less than 8% including less than 5% of such "irrelevant" amino acids.

In a further aspect of the invention there is provided a polypeptide or an analogue that is immunologically equivalent with the polypeptide, which is obtainable by the above method and having the antigenic characteristics as it is defined above.

In a preferred embodiment such a polypeptide has a bioreactivity in terms of positive reactions in the DTH test and/or the IFN-γ assay that is higher than the bioreactivity of the same polypeptide when it is expressed in a non-lactic acid bacterial host organism, the higher bioreactivity being determined by subjecting the polypeptide and the polypeptide expressed in the non-lactic acid bacterial host organism to identical DTH reaction or IFN-γ assay protocols using in each protocol a group of test individuals and recording the number of positive reactions.

In this context, the expression "non-lactic acid bacterial host cell" includes any bacterial species not belonging to the group of lactic acid bacteria as defined herein before such as *E. coli* and *Mycobacterium* spp., and any other non-

bacterial host cells such as eucaryotic cells including plant or animal cells and fungal cells.

Specifically, such a desirable higher bioreactivity is preferably such that the polypeptide, when it is tested against a polypeptide expressed in a non-lactic acid bacterial host organism using identical DTH reaction protocols including a group of test subjects or test animals, elicits at least one more positive reaction relative to the polypeptide expressed in a non-lactic acid bacterial host organism, such as at least two more positive reactions or even at least three more positive reactions. This higher bioreactivity may also be expressed as a percentage increase of positive reactions relative to the non-lactic acid bacterially produced polypeptide. Thus a higher bioreactivity is preferably at least 5% increase of positive reactions such as at least 10% or least 20%, e.g. at least 30% increase including at least 50% increase or even at least 100% increase.

It is an important objective of the present invention to provide a polypeptide that does not only have advantageous properties over the currently used PPD tuberculin skin test reagent with respect to being able to discriminate between lymphoid cells primed from tuberculosis and vaccination, respectively, that is, a polypeptide with a high specificity, but which polypeptide also has a sensitivity which is comparable to that of the PPD tuberculin. Accordingly, in an advantageous embodiment of the invention the polypeptide has a diagnostic sensitivity in terms of the number of positive reactions in the DTH test that is substantially equivalent to the sensitivity of the PPD tuberculin reagent, the sensitivity being determined by subjecting the polypeptide and the PPD tuberculin to identical DTH reaction protocols using in each protocol a group of test individuals and recording the number of positive reactions. In the present context, the expression "substantially equivalent" implies that, when the PPD tuberculin and the polypeptide of the invention is compared using identical protocols including 12 test animals or human beings for DTH skin test, the number of positive reactions elicited by the polypeptide is at the most 4 lower, preferably at the most 3 lower and most preferably at the most 2 lower.

One object of the present invention is to provide a lactic acid bacterium in which the expression of the mycobacterial polypeptide of the invention under the control of a promoter region comprising at least one regulatory sequence or signal is inducible and/or regulatable by one or more environmental or growth condition factors and in which the regulatory sequence or signal is altered or modified.

Thus, in a further aspect the invention pertains to an expression vector capable of being replicated in a lactic acid bacterial cell, the vector comprising a promoter region and operably linked hereto a DNA sequence coding for a bioreactive polypeptide or analogue hereof as defined above and having the antigenic characteristics as also defined above.

In one preferred embodiment of the invention, the expression vector according to the invention comprises in its promoter region a promoter sequence element, the activity or function of which is inducible and/or regulatable by the presence/absence or the concentration of one or more environmental or growth condition factors associated with conventional lactic acid bacterial industrial production methods. In the present context, the expression "promoter sequence" is used in the conventional sense to designate the site whereto RNA polymerase can be bound.

The promoter region may, in accordance with the invention, be derived from any bacterial cell, but in preferred embodiments it is derived from a lactic acid bacterial species including the species mentioned above and *Bifidobacterium* spp. In useful embodiments, the promoter region is derived from a promoter region of *Lactococcus lactis* including *Lactococcus lactis* subspecies *lactis,* e.g the strain designated MG1363 (this strain is also referred to in the literature as *Lactococcus lactis* subspecies *cremoris* (Nauta et al., 1996)), and *Lactococcus lactis* subspecies *lactis* biovar. *diacetylactis.* A naturally occurring inducible promoter region which can be modified in accordance with the invention may be isolated by any conventional method for identifying and isolating nucleotide

sequences comprising a promoter sequence and sequences having an effect on the activity of the promoter. Examples of such promoter regions which, in accordance with the present invention, are useful as starting materials are given in WO 94/16086 including a region comprising the promoter P170. Typically, such a promoter region starter material has a size which is in the range of 50 to 10,000 base pairs, such as in the range of 50 to 2000 base pairs including a range of 50 to 200 base pairs.

Preferably, the above environmental or growth condition factors are selected from pH, the growth temperature, the oxygen content, a temperature shift eliciting the expression of heat chock genes, the composition of the growth medium including the ionic strength/NaCl content, the presence/absence of essential cell constituents or precursors herefor, the growth phase of the bacterium or the growth rate of the bacterium.

It will be understood that when the promoter is one, the induction or regulation of which is controlled by one or more substances present in a conventional growth medium, substances which are not normally components of such media, such as antibiotics or bacteriocins are, in accordance with the invention, generally not included as environmental or growth condition factors.

The promoter region of the vector according to the invention comprises, as it is mentioned above, at least one further nucleotide sequence element, the position, orientation, presence and/or sequence of which element has a regulatory effect on the expression of a gene operably linked to the promoter region. As used herein, the expression "further nucleotide sequence" may include a sequence encoding a ribosome binding site, a transcription factor binding site, a repressor binding site, a site mediating attenuated or auto-regulated gene expression, a DNA sequence which can be transcribed into mRNA having an altered affinity for the ribosome or an altered affinity for nucleases, a DNA sequence comprising a transcription terminus, or any other sequence capable of modulating and/or enhancing gene expression. In the present context, this term will also include DNA sequences in the promoter region

which has no specifically recognised function, such as e.g. sequences located between or adjacent to -10 and -35 promoter sequences and other consensus sequences.

In accordance with the invention, the position, orientation, presence and/or sequence of at least one of said promoter sequences and further nucleotide sequence elements of the expression vector may in other useful embodiments be modified relative to the position, orientation, presence and/or sequence of its corresponding non-modified element.

Thus, contemplated modifications of the sequences of the promoter region include any modification hereof which affects the frequency of transcription initiation. This is obtained by substitution, deletion, addition and/or insertion of one or more nucleotides using any conventional technique for that purpose including random or site-directed mutagenesis to provide e.g. point mutations e.g. by using PCR or a transposable element.

Further modifications of the promoter region may, in accordance with the invention, also be made in one or more of the above further nucleotide sequences using any of the above techniques, such as it will be explained in details in the following examples.

It will be understood that it is possible to provide an expression vector according to the invention wherein both a promoter sequence and a further nucleotide sequence is modified.

In one preferred embodiment, the vector according to the invention is one wherein the modification of at least one of the above elements results in that the expression of a gene which is operably linked to the promoter region is altered relative to the expression of the same gene under the control of the non-modified promoter region. In the present context, the expression "altered expression" is used to indicate that the amount of the gene product being produced is different from the amount of gene product produced when using,

under essentially identical environmental or growth conditions, a bacterium comprising the same gene under the control of a corresponding non-modified promoter region from which the modified promoter region is derived.

In one preferred embodiment, the above modification of a promoter sequence element and/or a further nucleotide sequence element results in the expression of the gene which is under the control of the modified promoter region being enhanced, resulting in an increased amount of gene product being produced as compared to the amount being produced by a bacterium in which the same gene is under the control of the non-modified promoter region from which the modified promoter region is derived. Preferably, the production of the gene product is increased at least two-fold, more preferably at least three-fold, even more preferably at least four-fold. Even substantially higher increases can be obtained such as at least five-fold, more preferably at least ten-fold and even more preferably at least fifty-fold including at least hundred-fold such as at least two hundred-fold increase.

The expression vector according to the invention comprises at least one restriction site for the insertion of the DNA sequence coding for the mycobacterial polypeptide so as to have the expression of the coding sequence under the control of the modified promoter region, i.e. the gene is inserted so that it is operably linked to the promoter region. It may be advantageous that the gene product as expressed by the inserted gene is translocated to the outside of the cell membrane or even released into the cultivation medium. This requires that the gene is preceded by a nucleotide sequence encoding a signal peptide functionality or that the gene is part of a hybrid sequence coding for a fusion protein which is secretable as the result of the fusion partner being provided with a leader sequence. Accordingly, the coding sequence which is inserted into a vector according to the invention may, if required, comprise such a nucleotide sequence coding for a signal peptide, i.e. a signal sequence. The signal peptide may be functionally linked to a propeptide.

In a further useful embodiment, the vector is one wherein the promoter region comprises (a) a promoter sequence element the function of which is regulatable by a factor selected from the group at least consisting of pH, the growth temperature, the oxygen content, a temperature shift eliciting the expression of heat chock genes, the composition of the growth medium including the ionic strength/NaCl content, the presence/absence of essential cell constituents or precursors herefor, and the growth phase of the bacterium, the growth rate of the bacterium and (b) at least one further nucleotide sequence element, the position, orientation, presence and/or sequence of which element has a regulatory effect on the expression of a gene operably linked to the promoter region,

the position, orientation, presence and/or sequence of at least one of said elements (a) or (b) being modified relative to the position, orientation, presence and/or sequence of the corresponding non-modified element.

In a further aspect, the invention provides a recombinant lactic acid bacterium selected from any of the species mentioned above which is transformed with the vector as defined above.

As described above, the polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the tuberculosis complex (*Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*) are capable of reacting is useful as a diagnostic reagent for detection of tuberculosis. Accordingly, the invention provides a diagnostic composition comprising the polypeptide, which can be applied for *in vivo* and *in vitro* testing. Whereas such a composition can contain only one polypeptide according to the invention, it may be advantageous to provide a diagnostic composition containing at least two different diagnostically active polypeptides. It has been recognised that a bioreactive polypeptide of the invention may not provide 100% efficacy with respect to identifying those individuals in a population which are suffering from tuberculosis or infections with other mycobacteria, but the proportion of the infected population which

is detected by one particular bioreactive polypeptide may be different for different polypeptides.

It will therefore be appreciated that by combining in a diagnostic composition according to the invention, a multiplicity of bioreactive polypeptides each identifying a different proportion of infected individuals in a population it is possible to reduce the number of false negative reactions and obtain an efficacy approaching 100%, such as an efficacy of detection that is at least 50%, such as at least 60%, including at least 75%, including at least 90% efficacy.

In useful embodiments, such a multi-polypeptide diagnostic composition contains a mixture of monomeric polypeptides, a mixture of at least one monomeric polypeptide and at least one multimeric polypeptide or a mixture of at least two different multimeric polypeptides, such as 3, 4, 5, 6, 7, 8, 9 or 10 multimereic polypeptides.

Besides the mycobacteria of the *M. tuberculosis* complex, infections in humans and animals may also be caused by other mycobacteria. Thus, as examples *M. leprae* is a well-known human pathogen and *M. avium* may infect poultry and other animals and occasionally human beings. Additionally, several other mycobacterial species including those mentioned above may be "opportunistic" pathogens in the sense that, whereas they do not normally cause infections in healthy individuals, they may infect "weak" individuals such as individuals having a reduced immune defence including HIV patients and humans subjected to an immunosuppressive treatment or cancer treatment.

It is therefore another objective of the invention to provide bioreactive polypeptides which are derived from a *Mycobacterium* species not belonging to the *M. tuberculosis* complex, and immunologically equivalent analogues, variants or modifications thereof. Such polypeptides are useful in the diagnosis of infections with the above mycobacterial species.

Accordingly, the invention provides in a further aspect a method as defined above of producing such polypeptides. The method is carried out *mutatis mutandi* as it is described herein for the bioreactive polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the *M. tuberculosis* complex are capable of reacting.

It may be advantageous to provide such a polypeptide which does not give rise to a diagnostic reaction in an individual being infected with a mycobacterium of the *M. tuberculosis* complex. Accordingly, in one useful embodiment, the polypeptide obtained is a polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the *M. tuberculosis* complex are not capable of reacting.

The polypeptide derived from a mycobacterial species other than those belonging to the *M. tuberculosis* complex can be in any of the forms as described herein for the *M. tuberculosis* complex-derived polypeptides such as monomers, multimers, homopolymers or heteropolymers as defined hereinbefore and they may have a bioreactivity as defined above including the capability to elicit a positive delayed type hypersensitivity (DTH) reaction in a DTH skin test and the capability to stimulate *in vitro* IFN-γ release from lymphocytes.

Like the polypeptides derived from an organism belonging to the *M. tuberculosis* complex, the non-*M. tuberculosis* complex-derived polypeptides are useful in diagnostic compositions optionally comprising conventional additives such as a stabilising agent, a preserving agent, an adjuvant and/or a salt. Other useful additives include agents that promotes penetration of the bioreactive polypeptide such as e.g. dimethyl sulphoxide (DMSO).

In an interesting aspect, the non-*M. tuberculosis* complex-derived polypeptide according to the invention is used in a vaccine composition which, if desired, contains an adjuvant compound.

The invention is further illustrated in the following non-limiting examples and the drawing wherein

Figure legends

Fig. 1 shows a SDS-PAGE (left) and the corresponding western blot (right) obtained from strain AMJ715 expressing MPT64. The SDS-PAGE shows a major distinct band of about 30 kDa; a protein band hybridizing at the same position in the western blot analysis confirms the identity of MPT64. MPT64 has been described to migrate at a position corresponding to 24 kDa (Oettinger et al., 1995). This discrepancy might be caused by the 16% tricine gels used in this study, as the native MPT64 from short-term filtrates of *M. tuberculosis* migrates at the same position (data not shown). Based on the amount of MPT64 antigen found in short-term filtrates (about 5%), the secreted amount of MPT64 antigen in *L. lactis* strain AMJ715 is estimated to 10-15 mg/l,

Fig. 2 shows a SDS-PAGE (left) and the corresponding western blot (right) obtained from strain AMJ700 expressing a single copy of ESAT-6. No obvious protein band could be identified on the Coomassie stained gel, but using a monoclonal ESAT-6 antibody, two major products at 6 kDa were visible. The same pattern was observed in short-term culture filtrates from *M. tuberculosis* expressing ESAT-6 (Sørensen et al., 1995). In order to quantify the amount of secreted ESAT-6 antigen, a recombinant ESAT-6 protein of known concentration produced in *E. coli* at Statens Seruminstitut Denmark (unpublished data), was used as standard. The protein produced by *E. coli* contains 24 artificial amino acids in the N-terminal due to a histidine tag and a multiple cloning site. Therefore the calculation is only semi-quantitative. Estimated from scanning the western blot, the secreted level of ESAT-6 antigen from strain AMJ700 was less than 5 mg/l,

Fig. 3 shows a SDS-PAGE (left) and a western blot (right) of supernatant from strain AMJ717 expressing two ESAT-6 gene copies in tandem. On the SDS-PAGE a dominant band having a molecular weight of about 30 kDa was

identified. Western blot analysis confirmed the identity of this band as having ESAT-6 epitopes. Using recombinant *E. coli* produced ESAT-6 protein as standard, the amount of secreted ESAT-6-ESAT-6 protein from strain AMJ717 was estimated to be in excess of 30 mg/l,

Fig. 4 illustrates the DTH reactions in naive Dunkin Hartley guinea pigs injected with PPD tuberculin (positive control), ESAT-6 produced in *E. coli* and ESAT-6 homodimer produced in *Lactococcus lactis*, respectively, and

Fig. 5 illustrates the DTH reactions in Dunkin Hartley guinea pigs pre-sensitized with *Mycobacterium tuberculosis* strain H37Rv injected with PPD tuberculin (positive control), ESAT-6 produced in *E. coli* and ESAT-6 homodimer produced in *Lactococcus lactis*, respectively.

Examples

EXAMPLE 1 Minimising a DNA fragment containing the* Lactococcus lactis *P170 promoter

The pH regulated promoter P170 from *Lactococcus lactis* MG1363 is located on a 9.7 kp DNA fragment (Israelsen et al., 1995), which also includes a small fragment of Tn*917* DNA originating from the rescue of P170 from the *L. lactis* MG1363 chromosome. The Tn*917* DNA fragment was found to interfere with the transcription directed by P170 and was subsequently eliminated by subcloning of P170.

A DNA fragment comprising P170 was PCR amplified from the *L. lactis* MG1363 chromosome using the primers designated 170 *Bam*HI and 170p3R, which are complementary, respectively, to the DNA sequence located immediately upstream the Tn*917* insertion point, and a sequence located 623 bp further upstream of this point. This amplified DNA fragment contains an *Eco*RV site located 205 bp upstream of the Tn*917* insertion point and a *Bam*HI site located in the 5' end of primer 170 *Bam*HI. The PCR fragment was digested with *Eco*RV and *Bam*HI, and a 205 bp fragment was inserted into pKS Bluescript II digested with *Eco*RV and *Bam*HI, generating pSMA470. pSMA470 was subsequently digested with *Xho*I (multiple cloning site) and *Bam*HI, and the 205 bp fragment comprising P170 DNA sequence was cloned into the promoter cloning vector pAK80 (Israelsen et al., 1995), generating plasmid pAMJ547.

The β-galactosidase activity expressed from this strain was subsequently determined (Miller, 1972) in samples obtained from corresponding sets of cultures grown overnight in either GM17 medium or ArgM17 medium.

The enzyme activities (Table 1.1) show that both promoter activity and pH regulation are contained on the 205 bp DNA fragment originating from integrant PA170.

pAMJ538 was constructed by exonuclease BAL31 deletions and comprises a 150 bp DNA fragment located immediately upstream the Tn*917* insertion point in integrant strain PA170. No promoter activity was associated with this 150 bp DNA fragment.

To further define the 5' boundary of the promoter region, PCR was used to amplify *L. lactis* MG1363 chromosomal DNA fragments that covered 150 bp (primer 170-150), 160 bp (primer 170-160), 170 bp (primer 170-170), 180 bp (primer 170-180), and 190 bp (primer 170-190) upstream the insertion point, respectively. These five gene specific primers, which all contain a *Xho*I restriction site in the 5' end, were used in combination with primer 170 *Bam*HI. The amplified DNA fragments were subsequently digested with *Xho*I and *Bam*HI and cloned into pAK80, generating pAMJ551, pAMJ552, pAMJ553, pAMJ554 and pAMJ561, respectively. The plasmids were introduced into *L. lactis* MG1363 and β-galactosidase activity assays (Miller, 1972) were performed on sets of cultures grown overnight in GM17 medium and ArgM17 medium, respectively. Based on the enzyme activity data (Table 1.1), the 5' end of the promoter region is located between bp No. 160 and bp No. 170 upstream of the Tn*917* insertion point. pH regulation was intact for the clone comprising the 170 bp P170 DNA fragment.

The P170 promoter region was subsequently minimised from the 3' end by using an approach similar to that described above. Primers complementary to the DNA sequence located, respectively, 20 bp (primer 170 p1 *Bam*HI), 40 bp (primer 170 p2 *Bam*HI), 60 bp (primer 170 p3 *Bam*HI), 80 bp (primer 170 p4 *Bam*HI), 100 bp (primer 170 p5 *Bam*HI), 110 bp (primer 170 p6 *Bam*HI), 120 bp (primer 170 p7 *Bam*HI), and 130 bp (primer 170 p8 *Bam*HI) upstream of the Tn*917* insertion point were used in PCR reactions together with the primer

170-170 (Fig. 3). The PCR amplified *L. lactis* MG1363 chromosomal DNA fragments were digested with *Xho*I and *Bam*HI and inserted into pAK80, generating the plasmids pAMJ569, pAMJ568, pAMJ567, pAMJ566, pAMJ565, pAMJ577, pAMJ578, and pAMJ579, respectively. The plasmids were introduced into *L. lactis* MG1363 and β-galactosidase activity assays were performed (Miller, 1972) on sets of cultures grown overnight in GM17 medium and ArgM17 medium, respectively (Table 1.1). The enzyme activities show that the 3' end of the promoter region is located between bp No. 120 and bp No. 130 upstream of the Tn*917* insertion point.

The data show that a minimal promoter region able to direct gene expression comprises the DNA fragment extending from bp No. 120 to bp No. 170 upstream of the Tn*917* point of insertion. This minimal promoter region is also regulatable by pH.

Table 1.1. β-Galactosidase activities (Miller Units) in strains of *L. lactis* grown overnight in GM17 (pH about 5.5 after growth) or ArgM17 (pH about 7.0 after growth)

	β-Galactosidase activity	
Lactococcus lactis strain	GM17	ArgM17
AMJ547	4.3	0.9
AMJ561	3.5	0.2
AMJ554	3.1	0.2
AMJ553	3.2	0.1
AMJ552	0.5	0.3
AMJ551	0.4	0.1
AMJ569	3.1	0.6
AMJ568	1.3	0.1
AMJ567	30	1.4
AMJ566	18	1.1
AMJ565	205	3
AMJ577	48	3
AMJ578	7	0.9
AMJ579	0.6	0.8
AMJ586	700	13

EXAMPLE 2 Construction of a series of pH regulatable gene expression/-secretion vectors for **L. lactis** ***based on the P170 promoter regions comprised in pAMJ553, pAMJ567 and pAMJ586***

A set of pH regulatable expression/secretion vectors was constructed by combining different P170 derivatives with DNA encoding the Usp translocation signal peptide contained in plasmid pNZ1020 (van Asseldonk et al., 1990, GenBank M35374). Essentially, the open reading frame of the *lac*LM cassette were deleted from the vectors pAMJ553, pAMJ567, and pAMJ586 and replaced by a novel PCR generated gene cassette containing DNA fragments encoding the Usp signal peptide, including the cleavage site for the signal peptidase, followed by a multiple cloning site. The Usp signal sequence was generated by using pNZ1020 as a template in a PCR reaction with primers designated Usp primer 1 and Usp primer 2. Usp primer 1 is complementary to the DNA sequence comprising the unique *Bam*HI site and the ATG start codon of *lac*LM on pAK80, and by the first 23 bp of the Usp45 signal sequence. Usp primer 2 is complementary to the four codons located immediately upstream of, and the two codons located immediately downstream of the signal peptidase cleavage site, followed by a multiple cloning site that includes *Bgl*II, *Pst*I and *Sal*I sites. The PCR generated DNA fragment comprises the ribosome binding site of *lac*LM followed by the first 29 codons of the 5' end of the Usp signal sequence and a multiple cloning site. The generated 158 bp PCR product was digested with *Bam*HI and *Sal*I and ligated into pAMJ553, pAMJ567, and pAMJ586, respectively, which had been digested with *Bam*HI and *Sal*I. After transformation into *E. coli*, plasmid DNA was purified and each construction was verified by DNA sequencing. The resulting vectors, facilitating pH regulatable expression of genes of interest, followed by secretion of the gene products to the culture medium, were termed pSMA607, pSMA609, and pSMA610, respectively.

EXAMPLE 3 Cloning and expression of two **Mycobacterium tuberculosis** ***antigens, MPT64 and ESAT-6, in the secretion vector pSMA610.***

In this Example is described the cloning and expression of two *Mycobacterium tuberculosis* antigens using the pH regulated secretion vector pSMA610. The

two antigens are proteins found in short-term culture filtrates of *M. tuberculosis*. The antigens are of potential use as diagnostic skin test reagents and as components in subunit vaccines directed against tuberculosis.

1. Cloning of the MPT64 antigen

The DNA sequence encoding the mature secreted antigen MPT64 (205 amino acids, 22.4 kDa) (Oettinger and Andersen, 1994) from the *M. tuberculosis* H37Rv (ATCC 27294) strain was amplified using PCR technique. The primers mpt64 p3 (5' TTTCTGCTGCAGCCCCGTTGTCAGGTGTTTACGCTGCGCCCAAGACCTACTG CGAG 3') (SEQ ID NO: 7) and mpt64 p2 (5' ACGCGTCGACCTAGGCCAGCATCGAGTC 3') (SEQ ID NO: 8) were used in a PCR reaction with chromosomal DNA from *M. tuberculosis* H37Rv as template (the *Pst*I and *Sal*I restriction sites in the primers are underlined). Following digestion with *Pst*I and *Sal*I, the amplified DNA fragment was ligated into the *Pst*I and *Sal*I sites of the secretion vector pSMA610, resulting in plasmid pAMJ715. This cloning was designed to obtain a fusion of the 27 N-terminal amino acids of Usp45, encoding a signal peptide, to the N-terminal of the mature MPT64 antigen.

The primary protein structure of the MPT64 antigen secreted from *L. lactis* MG1363 containing plasmid pAMJ715 should be identical to the primary protein structure of MPT64 found in short-term culture filtrates of *M. tuberculosis* strain H37Rv. Plasmid pAMJ715 was introduced into *L. lactis* and the resulting strain was named AMJ715.

2. Cloning of ESAT-6

Using the same approach as described above, another secreted antigen, ESAT-6 (95 amino acids, 6 kDa) (Sørensen et al., 1995) from *M. tuberculosis* strain H37Rv, was PCR amplified and inserted into the secretion vector pSMA610. The two primers MT ESAT-6-3 (5' GGAAGATCTATGACAGAGCAGCAGTGG 3') (SEQ ID NO: 9) and MT ESAT-6-2 (5' ACGCGTCGACCTATGCGAACATCCC 3') (SEQ ID NO: 10) were used in a PCR reaction with chromosomal DNA from *M. tuberculosis* H37Rv as template

(the *Bgl*II and *Sal*I restriction sites in the primers are underlined). The generated PCR fragment was digested with *Bgl*II and *Sal*I and ligated into pSMA610, predigested with *Bgl*II and *Sal*I, yielding plasmid pAMJ700.

Plasmid pAMJ700 was introduced into *L. lactis* resulting in strain AMJ700. Strain AMJ700 was designed to secrete an ESAT-6 antigen containing four artificial amino acids in the N-terminal of the antigen, originating from the multiple cloning site of pSMA610.

In another construction, two copies of the ESAT-6 gene were inserted in tandem into the secretion vector pSMA610. This was done by insertion of another copy of ESAT-6 into vector pAMJ700 that already contained one copy of the ESAT-6 gene. The second ESAT-6 gene copy was produced using the primers MT ESAT-6-3 and MT ESAT-6-4 (5' GGAAGATCTTGCGAACATCCCAGTG 3') (SEQ ID NO: 11). MT ESAT-6-4 contains a BglII restriction site (underlined) and is complementary to the C-terminal of ESAT-6 except for the lack of a stop codon. After PCR amplification, the DNA fragment was digested with *Bgl*II and inserted into pAMJ700 which was predigested with *Bgl*II. A plasmid containing two ESAT-6 gene copies inserted in the correct orientation was obtained and named pAMJ717. Plasmid pAMJ717 was designed to encode a secreted polypeptide composed of four artificial N-terminal amino acids and two artificial linker amino acids, originating from the multiple cloning sites between the two ESAT-6 copies. pAMJ717 was introduced into *L. lactis* resulting in strain AMJ717. Thus, if the ESAT-6 homodimer is assembled utilising other restriction enzyme sites, the linker sequence and N-terminal leader sequence would consist of other amino acids.

3. Fermentation of *L. lactis* strains AMJ700, AMJ715 and AMJ717 expressing mycobacterial antigens.

Strain AMJ700, AMJ715 and AMJ717 were pre-propagated in GM17 medium supplemented with 1 µg/ml erythromycin. 10 ml overnight culture of each strain was inoculated into separate fermenters, each containing 1 litre SAIV

medium (Jensen and Hammer, 1993) supplemented with 1 µg/ml erythromycin. The incubation temperature was kept at 30°C with constant stirring at 300 rpm. The pH value was monitored and a set point of 5.2 was kept by titrating with 1M NaOH. The pH set point was reached after 6-8 hours of growth and after additional 4 hours of incubation, samples were collected for analysis of antigen production in each strain.

The cell pellet was removed by centrifuging the cultures at 3000 x g for 10 min. The supernatants were prepared for further analysis by concentrating 40 times using the microfiltration system available from Amicon and Millipore.

4. Detection and quantification of mycobacterial antigens produced in *L. lactis* by SDS-PAGE and western blotting.

Concentrated culture supernatants from strain AMJ700, AMJ715 and AMJ717 were boiled in sample buffer (Novex) containing 5% DTT prior to loading onto 16% tricine polyacrylamide SDS-gels (Novex). Samples were subjected to electrophoresis and the gels were stained with colloidal Coomassie Blue using the protocols recommended by Novex (Figs 1-3).

Proteins were electroblotted onto a nitrocellulose membrane (Novex) using the Novex mini-tank blotter for 1.5 hours with a constant power of 50 Volts. The membrane was blocked for 5 min. in TBS buffer (50 mM Tris-base pH 10.2, 500 mM NaCl) containing 5% BSA, washed once in TBS buffer containing 0.05% Tween-20 and 0.1% BSA. The membrane was subsequently incubated overnight with the primary antibody corresponding to the respective antigens.

Monoclonal antibody HYB76-8 (Klausen et al., 1994) directed against ESAT-6, and C24b1 (Andersen et al., 1991) directed against MPT64 were used at a dilution of 1:500 in TBS buffer containing 0.05% Tween-20 and 0.1% BSA. The membranes were washed three times for 5 minutes in the same buffer as used for primary antibody incubation. The secondary rabbit anti-mouse AP-conjugated antibody (DAKO) was diluted 1:2000 and incubated with the membrane for 2 hours at the same conditions as for the primary antibody. The

previous washing conditions were introduced and finally the nitrocellulose membrane was developed using the NBT/BCIP Ready-to-Use tablets as recommended by the manufacturer (Boehringer Mannheim).

EXAMPLE 4 Production of the polypeptide comprising 2 copies of ESAT-6 in 10 L production scale

The *Lactococcus lactis* strain AMJ717 was cultivated anaerobically in a fermenter containing 10 L SAIV medium at pH about 6.5. The fermentation was performed at about 30°C for about 15 hours. Following fermentation, the lactic acid bacterial cells were separated from the medium, and the resulting supernatant was concentrated about 50 times by cross-flow filtration.
Tween 20 is added to the culture supernatant to a final concentration of 0.01 %(v/v) . The culture supernatant is concentrated to approx. 1/50 by volume using a prep-scale filter with a molecular weight cut off of 10 kDa.

The concentrated supernatant is then extensively dialysed against 20 mM Bis-Tris pH 5.5; 0.01% (v/v) Tween-20 using diafiltration and aliqouted into vials containing 50 mL. The concentrated and diafiltrated supernatant is stored at -80°C until use.

The concentrated and diafiltrated supernatant is thawed and the ESAT6-ESAT6 can be purified using the following protocol.

Size exclusion chromatography is performed using a S200 (5 x 100 cm) column equilibrated with 20 mM Bis-Tris pH 5.5; 150 mM NaCl. Fractions containing specific protein are pooled and diluted with 20 mM Bis-Tris pH 5.5 until a conductivity < 5 mS/cm is reached.
The diluted sample is then loaded on an anion exchange column packed with Q-sepharose (2.6 x 5 cm). After application, the column is washed using 20 mM Bis-Tris pH 5.5; 50 mM NaCl and specific protein is eluted using a salt gradient (50-500 mM NaCl diluted in the washing buffer).

Fractions containing specific protein is then concentrated using anion exchange chromatography using the following protocol:
Dilute pooled fractions with 20 mM Bis-Tris pH 5.5 until a conductivity < 2.5 mS/cm is reached.
The sample is loaded onto an anion exchange column (Q-sepharose 1x2 cm) equilibrated in 20 mM Bis-Tris pH 5.5. After application, the column is washed with 20 mM Bis-Tris pH 5.5; 50 mM NaCl.
Specific protein is obtained by elution using 20 mM Bis-Tris pH 5.5; 500 mM NaCl.

The described process of purification does not include denaturation by e.g. urea and subsequent dialysis, as it is known from the purification of other protein products. The proteins will not be denaturated and subsequently renaturated with the described purification process. This causes the proteins to maintain their native conformation, and thus more natural epitopes. This also renders the product more reproducible from batch to batch.

The described process of purification also makes the his-tag sequence as used in the purification of the *E. coli* Esat-6 and known from other purification processes, unnecessary. The lack of a substantial N-terminal leader sequence in the polypeptide product is anticipated to ensure that epitopes are not covered by the N-terminal leader sequence.

EXAMPLE 5 The bioreactivity of the ESAT-6 polypeptide

The partially purified and concentrated polypeptide obtained in Example 4 was tested for its efficacy in a DTH reaction skin test in guinea pigs infected with *Mycobacterium tuberculosis* strain H37Rv. PPD tuberculin was used as a positive control in the experiment and as negative controls naive animals were included. For comparative purposes, a ESAT-6 polypeptide expressed in *E. coli* was included.

Four weeks prior to the skin test experiments, 12 Dunkin Hartley guinea pigs were infected by the intravenous route. The infection dosage per animal was 0.2 ml of a suspension of the bacteria containing 2.5 x 10^5 CFU/ml.

The antigen preparations used in the skin tests were as follows:

(i) A commercial PPD preparation which is a solution of the protein (100 tuberculin units/ml) in PBS containing 0.005% polysorbate 80 and 0.01% chinisol (PBSTC),

(ii) The purified and concentrated polypeptide preparation from Example 4 was diluted in 2xPBSTC and PBS to obtain a solution containing 0.005% polysorbate 80 and 0.01% chinisol and 20 µg/ml of the ESAT-6 tandem protein,

(iii) A preparation of ESAT-6 polypeptide produced in *E.coli* in PBSTC and containing 20 µg/ml of the polypeptide.

On day one, each of the antigen preparations were injected intradermally according to a randomization scheme into 12 infected and 6 naive guinea pigs, respectively. The dosage per animal was 100 µl of the respective preparations.

On day two, the diameter of the inflammatory skin reaction (erythema) was measured by means of a ruler. The diameter measurements were based on two readings made independently by 2 persons and the result was calculated as the means of the four readings. A positive reaction was recorded when the diameter of the reaction was $\geq$ 5 mm.

The results are summarised in Fig. 4 (naive animals) and Fig. 5 (infected animals). It appears that for all three antigen preparations, no reactions were observed in the naive animals. In the infected animals, a positive reaction was found with the PPD preparation in 12/12 guinea pigs. With the *E. coli*-expressed ESAT-6 preparation positive reactions were seen in only 6/12

guinea pigs whereas the *Lactococcus lactis*-expressed ESAT-6 fusion protein gave a positive reaction in 12/12 guinea pigs.

From the examples described above it is clearly demonstrated that the secretion vector pSMA610 is capable of expressing heterologous genes and subsequently direct the gene products to the external growth medium. The amount of secreted protein is variable, ranging from less than 5 mg/l to levels exceeding 30 mg/l. These differences might be caused by different chemical or physical characteristics of the individual heterologous proteins. The activity of the P170 promoter in the chemically defined media SAIV was also demonstrated. This activity is important due to the fact that many pharmaceutical proteins are not allowed to be produced in complex media containing animal proteins.

Furthermore, it was demonstrated that the tested lactic acid bacterially produced mycobacterial polypeptide had a bioreactivity which is substantially higher than that of a polypeptide produced in *E. coli* and which is equivalent to the bioreactivity of the PPD tuberculin.

EXAMPLE 6 Sensitivity and specificity of the ESAT-6 homodimer

The biological activity of ESAT6 was tested in a guinea pig model. The efficacy of ESAT6-ESAT6 produced in L. lactis to elicit a DTH like reaction in Mycobacterium tuberculosis infected Guinea pigs was investigated.
For comparative purposes a ESAT6- polypeptide expressed in E. coli (produced as described in Harboe et al. 1998) was included. The test was carried out according to the following protocol.

Four weeks prior to skin test experiments Dunkin Hartley guinea pigs were infected with Mycobacterium tuberculosis.
Guinea pigs were infected using i.v. infection (0.2ml 2.5 x 10^5 CFU/ml) using the Mycobacterium tuberculosis H37Rv strain or by aerosol infection using Mycobacterium tuberculosis Erdman strain at a dosage of 1 x 10^5CFU/ml

The clinical onset of the disease was followed by observing the animals during the infection period. Usually the animals appear ill. Piloerection and a weight loss of about 10% per week after two weeks of infection are clinical observations. The animals can be tested PPD positive or be tested for the appearance of granulomas in the lungs after the experiments.

A group of BCG-immunised guinea pigs was used as control.
Guinea pigs were immunised with 0.1 ml BCG-Danish 1331 four weeks prior to skin test experiments.

4 weeks after infection, skin tests were carried out using the same protocol as described in example 5.

Data representing experiments performed using 61 guinea pigs, 36 of which were infected with Mycobacterium tuberculosis and 25 guinea pigs were immunised using the BCG-danish strain 1331.

Considering DTH reactions larger that 7 mm as positive the following results were obtained:

	Tuberculin	coli-ESAT6	ESAT6-ESAT6
Sensitivity	**83%**	**74%**	**94%**
Specificity	**2%**	**95%**	**94%**

Considering DTH reactions larger that 10 mm as positive the following results were obtained:

	Tuberculin	coli-ESAT6	ESAT6-ESAT6
Sensitivity	**56%**	**44%**	**84%**
Specificity	**10%**	**100%**	**100%**

Thus, depending on the choice of cut-off for positive DTH responds, ESAT6-ESAT6 has a sensitivity being 20-40% better than that of ESAT6 polypeptide produced in E. coli.

The true value for positive prediction with this new antigen can only be determined through clinical use. Most important is to consider a cut-off value which ensures a high specificity without compromising sensitivity.

LIST OF REFERENCES

Andersen, Å. B., L. Ljungquist, K. Haasløv and M. W. Bentzon. 1991. Scand. J. Immunol. **34**:365-372.

de Ruyter, P.G.G.A., O.P. Kuipers, M.M. Beerthuyzen, I. van Alen-Boerrigter, and W.M. de Vos. 1996. J. Bacteriol. **178**:3434-3439.

Harboe et al., Infection and Immunity, Feb. 1998, p 717-723

Israelsen, H., S.M. Madsen, A. Vrang, E.B. Hansen, and E. Johansen. 1995. Appl. Environ. Microbiol. **61**: 2540-2547.

Jensen, P. and K. Hammer. 1993. Appl. Environ. Microbiol. **59**:4363-4366.

Klausen, J., M. Magnusson, Å. B. Andersen, and C. Koch. 1994. Scand. J. Immunol. **40**:345-349.

Miller, J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Nagai, S., H.G. Wiker, M. Harboe, M. Kinomoto. 1991. Infection and Immunity **59**:372-382.

Nauta, A., D. van Sinderen, H. Karsens, E. Smit, G. Venema, and J. Kok. 1996. Mol. Microbiol. **19**:1331-1341.

Oettinger, T. and A.B. Andersen. 1994. Infection and Immunity. **62**:2058-2064.

O'Sullivan, D.J., S.A. Walker, S.G. West, and T.R. Klaenhammer. 1996. Biotechnology **14**:82-87.

Steidler, L., J.M. Wells, A. Raeymaekers, J. Vandekerckhove, W. Fiers, and E. Remaut. 1995. Appl. Environ. Microbiol. **61**:1627-1629.

Sørensen, A.L., S. Nagai, G. Houen, P. Andersen and Å.B. Sørensen Å.B. 1995. Infection and Immunity **63**:1710-1717.

van Asseldonk, M., A. Simons, H. Visser, W.M. de Vos, and G. Simons. 1993. J. Bacteriol. **175**:1637-1644.

van Rooijen, R.J., M.J. Gasson, and W.M. de Vos. 1992. J. Bacteriol. **174**:2273-2280.

Wells, J.M., P.W. Wilson, P.M. Norton, M.J. Gasson, and R.W.F. Le Page. 1993a. Mol. Microbiol. **8**:1155-1162.

Wells, J.M., P.W. Wilson, P.M. Norton, M.J. Gasson, and R.W.F. Le Page. 1993b. Appl. Environ. Microbiol. **59**:3954-3959.

Yamaguchi, R., K. Matsuo, A. Yamazaki, C. Abe, S. Nagai, K. Terasaka and T. Yamada. 1989. Infection and Immunity **57**: 283-288.

CLAIMS

1. A method of producing a bioreactive polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the *M. tuberculosis* complex (*Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*) are capable of reacting, or an analogue that is immunologically equivalent to the polypeptide, the method comprising the steps of (i) providing a DNA sequence coding for said polypeptide, (ii) inserting said coding sequence into a vector capable of replicating in a lactic acid bacterium, (iii) transforming a lactic acid bacterium with said vector, (iv) cultivating the thus transformed bacterium in a culture medium under conditions where the polypeptide is expressed, and (v) harvesting the mycobacterial polypeptide,

the polypeptide thus obtained is capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in at least 10% of guinea pigs previously infected with virulent mycobacteria belonging to the *M. tuberculosis* complex, the DTH reaction essentially being carried out using the following protocol:

(i) an at least partially purified preparation of the polypeptide is diluted with PBS containing 0.005% polysorbate and 0.01% chinisol to obtain a polypeptide concentration of about 20 µg/ml,

(ii) 100 µl of the diluted polypeptide preparation is injected intradermally into a group of Dunkin Hartley guinea pigs infected i.v. 4 weeks prior to the DTH reaction test with the *Mycobacterium tuberculosis* strain H37Rv at a dosage of about 0.5×10^5 CFU, and

(iii) the size of the inflammatory reaction at the sites of injection is measured on day 2 by means of a ruler, a reaction of a size $\geq$ 5 mm being recorded as a positive reaction.

2. A method according to claim 1 wherein the vector comprises a signal sequence operably linked to the coding sequence whereby the polypeptide is secreted across the cell membrane.

3. A method according to claim 1 wherein the coding sequence for the polypeptide is derived from *Mycobacterium tuberculosis*.

4. A method according to claim 3 wherein the coding sequence is operably linked to a signal sequence whereby the polypeptide is secreted across the cell membrane.

5. A method according to claim 3 wherein the polypeptide is an immunologically active polypeptide carrying an epitope or an antigenic determinant, said polypeptide is capable of conferring immunity to infections with a bacterium of the *M. tuberculosis* complex when administered to an animal including a human being.

6. A method according to claim 2 or 4 wherein the polypeptide is secreted into the culture medium in an amount of at least 20 mg/L.

7. A method according to claim 1 wherein the coding sequence is part of a hybrid coding sequence whereby the polypeptide is expressed as part of a fusion protein.

8. A method according to claim 7 wherein the fusion protein comprises at least two polypeptides of mycobacterial origin.

9. A method according to claim 8 wherein the fusion protein comprises at least two polypeptides of mycobacterial origin having essentially the same amino acid sequence.

10. A method according to claim 7 wherein the fusion protein comprises at least two polypeptides of *Mycobacterium tuberculosis* origin.

11. A method according to claim 10 wherein the fusion protein comprises at least two polypeptides of *Mycobacterium tuberculosis* origin having essentially the same amino acid sequence.

12. A method according to claim 6 wherein the hybrid coding sequence is operably linked to a sequence coding for a signal peptide to provide secretion of the fusion protein across the cell membrane.

13. A method according to claim 1 wherein the lactic acid bacterium is *Lactococcus lactis*.

14. A method according to any of claims 1-13 wherein the polypeptide molecule as harvested is in a form where it contains less than 10% amino acids which are not naturally associated with the polypeptide.

15. A polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the *M. tuberculosis* complex are capable of reacting, or an analogue that is immunologically equivalent to the polypeptide, the polypeptide is obtainable by the method according to any of claims 1-13 and is capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in at least 10% of guinea pigs previously infected with virulent mycobacteria belonging to the *M. tuberculosis* complex, the DTH reaction essentially being carried out using the following protocol:

(i) an at least partially purified preparation of the polypeptide is diluted with PBS containing 0.005% polysorbate and 0.01% chinisol to obtain a polypeptide concentration of about 20 µg/ml,

(ii) 100 µl of the diluted polypeptide preparation is injected intradermally into a group of Dunkin Hartley guinea pigs infected i.v. 4 weeks prior to the DTH reaction test with the *Mycobacterium tuberculosis* strain H37Rv at a dosage of about 0.5×10^5 CFU, and

(iii) the size of the inflammatory reaction at the sites of injection is measured on day 2 by means of a ruler, a reaction of a size $\geq$ 5 mm being recorded as a positive reaction.

16. A polypeptide according to claim 15 which has a bioreactivity in terms of positive reactions in the DTH test that is higher than the bioreactivity of the same polypeptide when it is expressed in a non-lactic acid bacterial host organism, the higher bioreactivity being determined by subjecting the polypeptide and the polypeptide expressed in the non-lactic acid bacterial host organism to identical DTH reaction protocols using in each protocol a group of test individuals and recording the number of positive reactions.

17. A polypeptide according to claim 16 which, when tested against the polypeptide expressed in a non-lactic acid bacterial host organism using identical DTH reaction protocols, elicits at least one more positive reaction relative to the polypeptide expressed in a non-lactic acid bacterial host organism.

18. A method of producing a bioreactive polypeptide which is derived from a *Mycobacterium* not belonging to the *M. tuberculosis* complex, the method comprising the steps of (i) providing a DNA sequence coding for said polypeptide, (ii) inserting said coding sequence into a vector capable of replicating in a lactic acid bacterium, (iii) transforming a lactic acid bacterium with said vector, (iv) cultivating the thus transformed bacterium in a culture medium under conditions where the polypeptide is expressed, and (v) harvesting the mycobacterial polypeptide, said polypeptide being selected from the group consisting of a polypeptide with which lymphoid cells previously primed with mycobacteria are capable of reacting and an analogue of said polypeptide that is immunologically equivalent to the polypeptide.

19. A method according to claim 18 wherein the polypeptide obtained is a polypeptide with which lymphoid cells previously primed with mycobacteria belonging to the *M. tuberculosis* complex are not capable of reacting.

20. A method according to claim 18 or 19 wherein the *Mycobacterium* not belonging to the *M. tuberculosis* complex is selected from the group consisting of *M. avium, M. intracellulare, M. kansasii, M. scrufolaceum, M. marinum, M. ulcerans, M. fortuitum, M. chelonei, M. phlei, M. gordanae, M. smegmatis, M. paratuberculosis* and *M. leprae.*

21. A polypeptide that is obtainable by the method according to any of claims 18-20.

22. A polypeptide according to claim 21 which is capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in a DTH skin test.

23. A polypeptide according to claim 15 or 21 which is capable of stimulating *in vitro* IFN-γ release from lymphocytes.

24. A diagnostic composition comprising a polypeptide according to any of claims 15-17 or 21-23.

25. A vaccine comprising a polypeptide according to claim 15 or 21.

26. A vaccine according to claim 25 further comprising an adjuvant.

27. An ESAT-6 homopolymer polypeptide comprising at least two copies of ESAT-6 optionally linked with a linker sequence and optionally with an N-terminal leader sequence.

28. An ESAT-6 homopolymer polypeptide according to claim 27 comprising at least 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 copies of ESAT-6.

29. An ESAT-6 homodimer polypeptide according to claim 27 comprising 2 copies of ESAT-6.

30. An ESAT-6 homopolymer polypeptide according to claim 27 comprising a linker sequence with 1 amino acid.

31. An ESAT-6 homopolymer polypeptide according to claim 27 comprising a linker sequence with 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 amino acids.

32. An ESAT-6 homopolymer polypeptide according to claim 31 comprising a linker sequence with 2 amino acids.

33. An ESAT-6 homopolymer polypeptide according to claim 32 wherein the linker sequence is Arg-Ser.

34. An ESAT-6 homopolymer polypeptide according to any of claims 27-33, wherein the N-terminal leader sequence is Asp-Thr-Arg-Ser.

35. An ESAT-6 homopolymer polypeptide according to any of claims 27-34, wherein the polypeptide has a specificity of more than 70% such as 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% or such as 100%.

36. An ESAT-6 homopolymer polypeptide according to any of claims 27-35, wherein the polypeptide is capable of eliciting a positive delayed type hypersensitivity (DTH) reaction in at least 10% of guinea pigs previously infected with virulent mycobacteria belonging to the M. tuberculosis complex, the DTH reaction essentially being carried out using the following protocol:

(i) an at least partially purified preparation of the polypeptide is diluted with PBS containing 0.005% polysorbate and 0.01% chinisol to obtain a polypeptide concentration of about 20 µg/ml,

(ii) 100 µl of the diluted polypeptide preparation is injected intradermally into a group of Dunkin Hartley guinea pigs infected i.v. 4 weeks prior to the DTH

reaction test with the Mycobacterium tuberculosis strain H37Rv at a dosage of about 0.5 x 105 CFU, and

(iii) the size of the inflammatory reaction at the site(s) of injection is measured on day 2 by means of a ruler, a reaction of a size > 5 mm being recorded as a positive reaction.

37. An ESAT-6 homopolymer polypeptide according to claim 36, wherein the polypeptide has a sensitivity of more than 20%, such as 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, or 94%.

38. A method of producing a homopolymer according to any of claims 27-37, comprising the steps of (i) providing a DNA sequence coding for said homopolymer, (ii) inserting said coding sequence into a vector capable of replicating in a cell, (iii) transforming said cell with said vector, (iv) cultivating the thus transformed cell in a culture medium under conditions where the homopolymer is expressed, and (v) harvesting the homopolymer.

39. A method according to claim 38 wherein the cell is a lactic acid bacterium.

40. A diagnostic composition comprising an ESAT-6 homopolymer polypeptide according to any of claims 27-37.

41. An immunological agent comprising an ESAT-6 homopolymer polypeptide according to any of claims 27-37.

42. An immunological agent according to claim 41 further comprising an adjuvant.

43. An immunological agent according to claim 41 or 42, in the form of a vaccine.

44. An immunologic composition according to claim 41 or 42, which is in the form of a skin test reagent.

45. The use of an ESAT-6 homopolymer according to any of claims 27-37 or 40-44 in the preparation of a pharmaceutical composition for the diagnosis of or vaccination against tuberculosis caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum* or *Mycobacterium bovis*.

46. A vaccine for immunising an animal, including a human being, against tuberculosis, comprising as the effective component one or more copies of a DNA sequence encoding an ESAT-6 homopolymer such as an ESAT-6 homodimer, formulated in such a way that expression of the ESAT-6 homopolymer in the mammalian cell is promoted.

47. A vaccine for immunising an animal, including a human being, against tuberculosis, comprising as the effective component a non-pathogenic microorganism, wherein at least one copy of a DNA fragment comprising a DNA sequence encoding an ESAT-6 homopolymer has been incorporated into the genome of the microorganism in a manner allowing the microorganism to express and optionally secrete the polypeptide.

48. A vaccine according to claim 47, wherein the microorganism is a bacterium.

49. A vaccine according to claim 48, wherein the microorganism is *Mycobacterium bovis* BCG, such as *Mycobacterium bovis* BCG strain: Danish 1331.

50. A method for diagnosing ongoing or previous sensitisation in an animal or a human being with bacteria belonging to the tuberculosis complex, the method comprising providing a blood sample from the animal or human being, and contacting the sample from the animal with an ESAT-6 homodimer according to any of claim 27-37 or 40-44, a significant release into the

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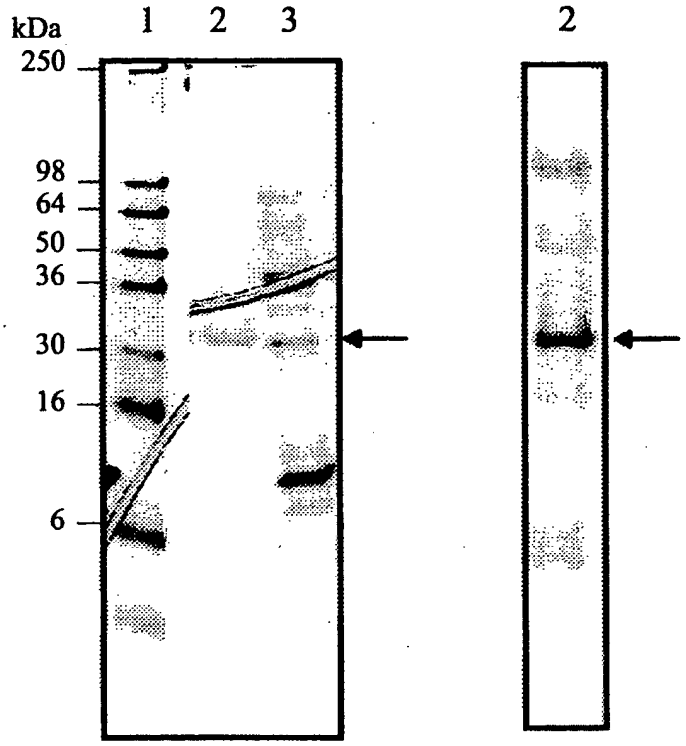

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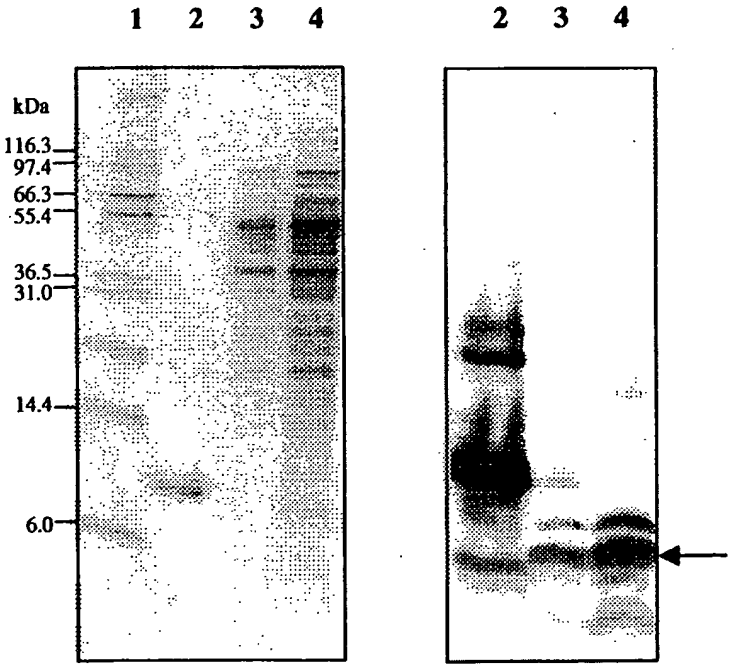

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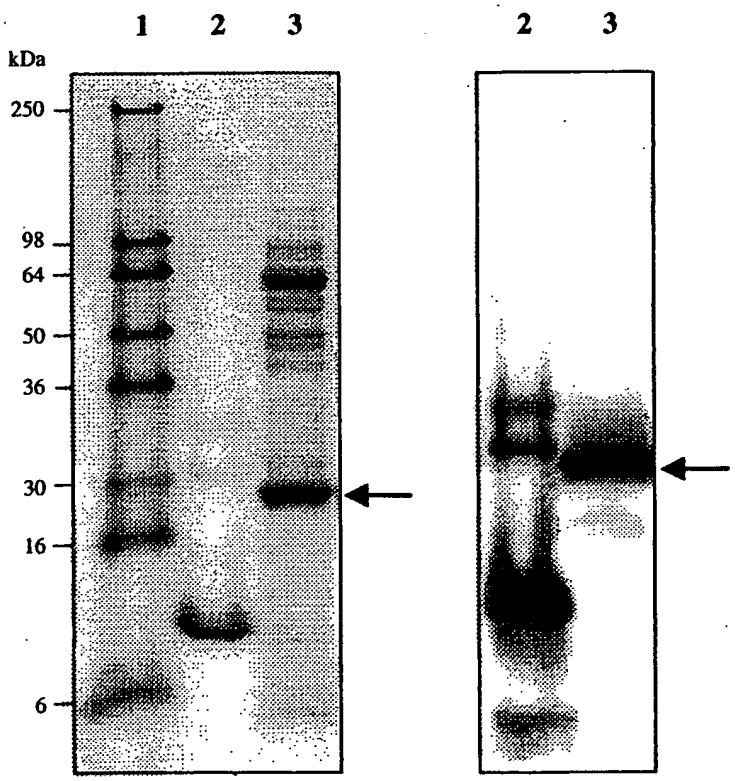

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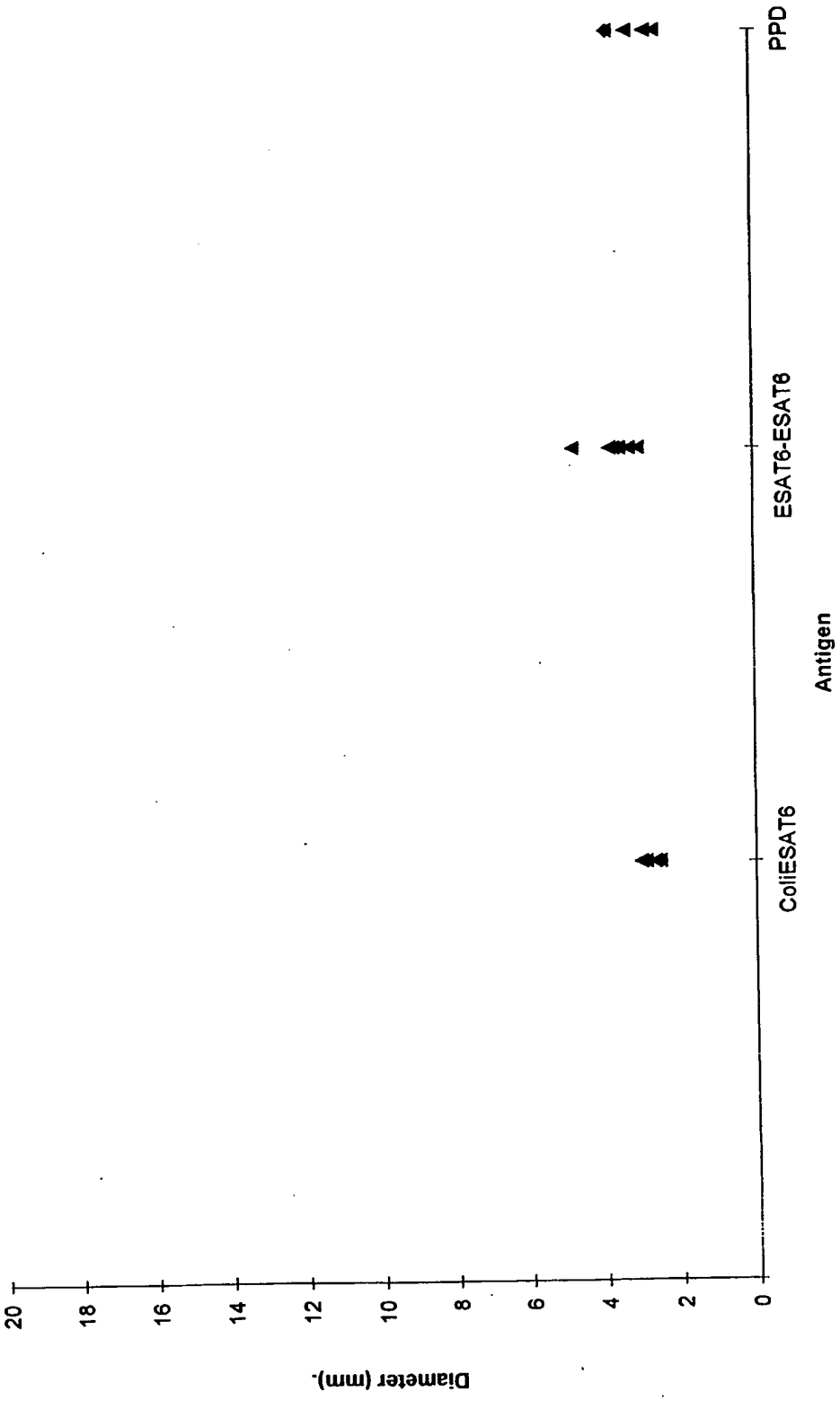
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Fig. 4

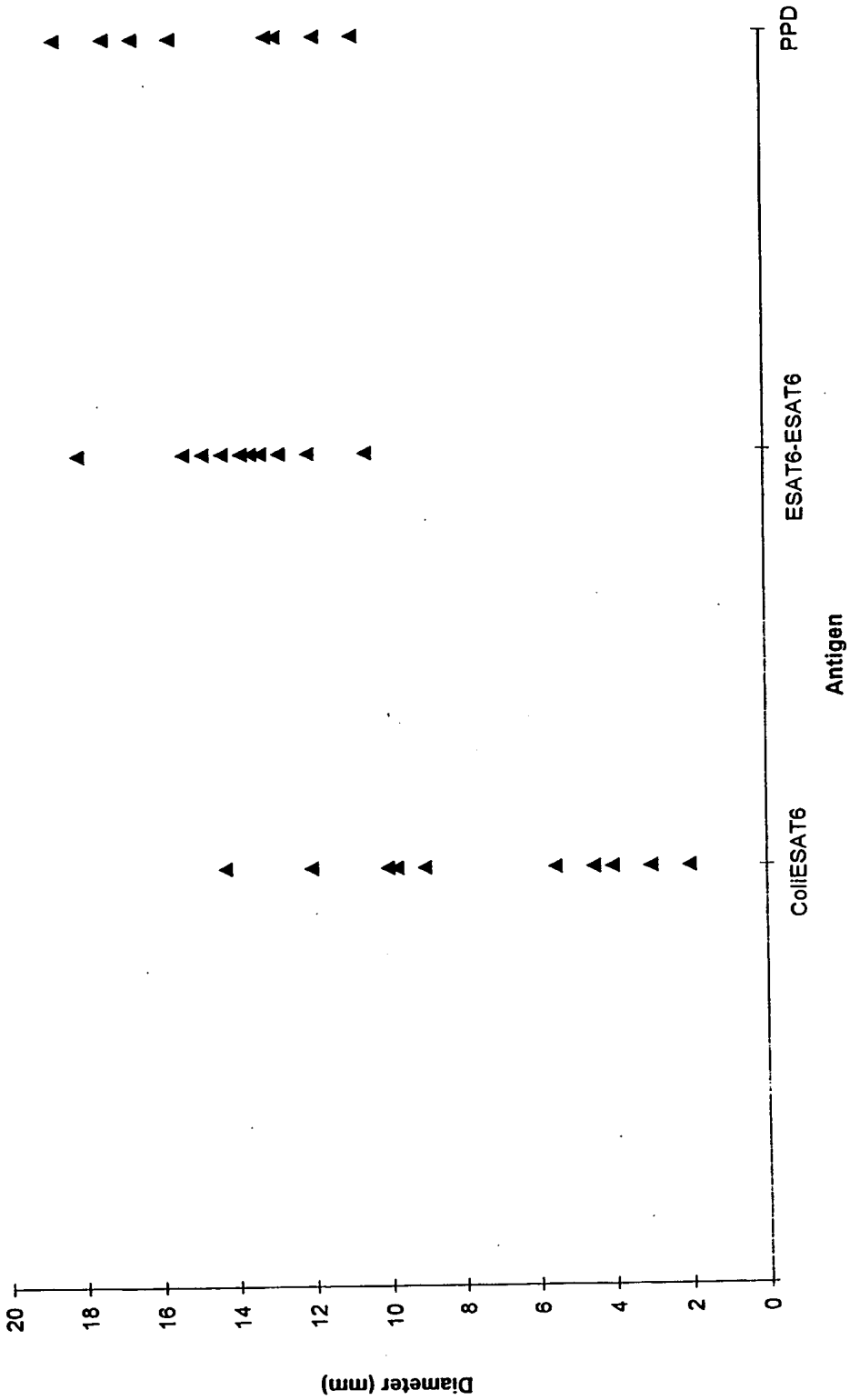
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Fig. 5

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